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(54) Title: POLYMERIC RESIN FOR DISULFIDE BOND SYNTHESIS

(57) Abstract

Disulfide resin compositions in which, following disulfide-thiol interchange, each thiol moiety remains attached to the resin, such resins affect the formation of inter or intramolecular peptide or protein disulfide bonds.

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POLYMERIC RESIN FOR DISULFIDE BOND SYNTHESIS

TECHNICAL FIELD

This invention relates to novel polymeric resins useful to effect the formation of peptide or protein disulfide bonds within or between molecules containing thiol groups.

BACKGROUND OF THE INVENTION

Disulfide bond formation plays a critical role in protein folding. Disulfide bonds also serve to impart or maintain structural stability and biological activity in peptides and proteins. The chemical synthesis of peptides containing one or more intramolecular disulfide bonds between pairs of cysteine residues usually requires the formation of these bonds after the peptide has been synthesized. The preferred method of forming intramolecular disulfide bonds requires use of aqueous media containing mild oxidizing agents which do not also oxidize methionine, histidine, tryptophan and tyrosine residues of the peptide.

For formation of single disulfide bonds in simple peptides, stronger, rapidly acting oxidants such as I_2 and $K_3Fe(CN)_6$ are sometimes used, but they give rise to kinetic control of product formation and may cause overoxidation. In addition, the peptide product must be separated from unreacted and reduced oxidant.

The two most commonly used methods for formation of peptide or protein disulfide bonds in aqueous solution under mild redox conditions are air oxidation and mixed thiol-disulfide interchange. Because these milder oxidations take place slowly, equilibration between conformers can occur to give thermodynamic control of product formation. Air oxidation usually requires a high dilution of the peptide or protein in neutral or basic solution and a long reaction time to complete disulfide bond formation. Unlike other methods of disulfide bond formation, air oxidation yields H_2O as a byproduct, enabling easy isolation of the peptide product.

Thiol-disulfide exchange reactions to form disulfide bonds between peptide cysteine pairs also require a high dilution of the peptide, but may be performed at basic, neutral or acid pH, depending upon the disulfide reagent selected. Unlike air oxidation, the peptide product of a disulfide interchange reaction must be separated from the disulfide reagent and its reduced product. A variety of disulfide reagents may be used, including oxidized glutathione and Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)].

The mechanism for reversible thiol-disulfide interchange reactions is shown in Figure 1. When a highly dilute dithiol undergoes thiol-disulfide interchange in the presence of excess disulfide component, an intramolecular disulfide is formed as shown in Figure 2. Thus, the formation of intramolecular disulfide bonds in peptides and proteins would be greatly aided by an essentially irreversible thiol-disulfide interchange reaction between a di- or oligothiol peptide or protein at a relatively high concentration in basic, neutral or acidic aqueous medium and a resin-bound disulfide substituent which forms thiones upon reduction. Furthermore, the use of resins in which the dithio-containing substituent is doubly attached via a functional group on each side of the sulfur atoms of the disulfide bond, followed by separation of the resin from the liquid phase, would permit easy isolation of the soluble peptide disulfide product free of reactants and by-products. Resins which can effect the formation of inter- or intramolecular peptide or protein disulfide bonds were apparently unknown prior to this invention.

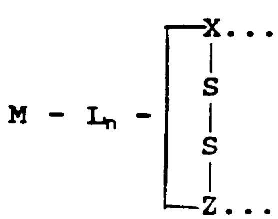
SUMMARY OF THE INVENTION

The present invention provides resins having a dithio-containing substituent. In one preferred embodiment, each moiety of the dithio substituent is covalently linked through a sulfur atom of the resin disulfide bond. The substituent is attached either directly to the resin or, in one

preferred embodiment, to the distal end of an extended linker moiety the proximal end of which is linked to the resin. By thiol-disulfide interchange, the resin-bound disulfide substituent brought in contact with a solution of a compound containing one or more thiol groups provides a means to form a disulfide bond within a polythiol, e.g., an oligothiol (intramolecular), or between two molecules of a monothiol (intermolecular) while concurrently producing one or preferably two resin-attached thione moieties arising from concomitant reduction of the resin-bound disulfide substituent.

The functional disulfide resin substituents that best serve this purpose are those that undergo thiol-disulfide interchange to yield two thiol moieties, each of which may convert to an energetically more stable thione tautomer and thus thermo-dynamically drives the reversible disulfide interchange essentially to completion. The reduction of thione-generating disulfides is shown schematically in Figure 3, where Y is an imino nitrogen, a thioether sulfur, an ether oxygen or a carbon atom that is double bonded to another substituent. Y and N in the first reaction scheme and the corresponding Y and C in the second may be part of a ring system.

Preferred embodiments of the invention include one or more monomeric or crosslinked disulfide moieties having the schematic formulae:



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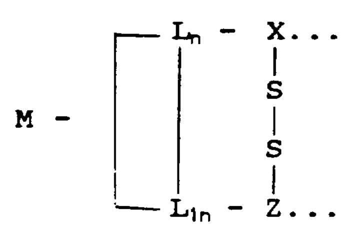


FORMULA I

FORMULA II

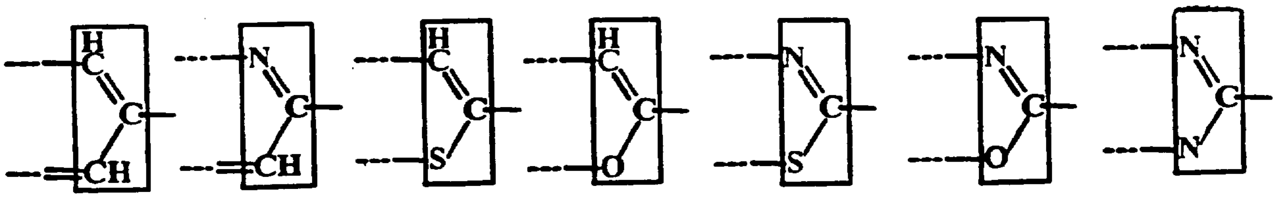
in which n is one or zero. The matrix M is any normally solid synthetic resin, i.e., a synthetic resin which has a melting

point below that at which reactions involving the resin are effected. More specifically, the melting point of matrix M is preferably at least 200°C. A matrix resin melting point range of 150°C. to 250°C. is appropriate. Specific matrix resins useful in the invention include, but are not limited to, polyolefins, polyacrylates, polymethacrylates, polystyrenes, 2 hydroxyethyl-methacrylate-ethylene dimethacrylate copolymers, styrene-divinyl benzene copolymer, styrene divinyl benzene copolymer onto which polyoxyethylene chains are grafted. M may also be a natural product, e.g., cellulose, or derivatized controlled pore glass.

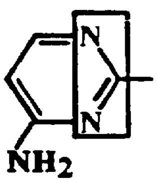
Each of L or L_1 has at least two chemically reactive groups attached to the matrix (M) and also to each of the X or Z moieties. L or L_1, which may be the same or different, are linkers effective to facilitate access of soluble reactants, such as thiols, to the disulfide bond. L or L_1 may be, for example, any straight or branched alkyl chain, a polyalkylene glycol chain, a peptide, or a polyvinyl alcohol chain. However, in Formula I moieties, L must be branched as shown. The linkers L or L_1 also may be crosslinked to provide resins having a plurality of Formula I or Formula II moieties. L or L_1 may be 5 to 5000 Angstroms, preferably 20-60, in length.

Each of the moieties X and Z is covalently joined either directly or by the linker L to the matrix M such that, following disulfide-thiol interchange, each of the resulting thiol moieties remains attached to the matrix M. See, e.g., Figure 4. Broken lines after the X and Z moieties indicate available covalent crosslinks with other Formula I or Formula II moieties.

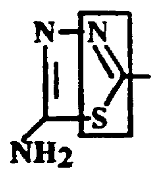
The X and Z moieties may be the same or different. In its generic scope, the invention includes, but is not limited to, the following representative X or Z structures in which the functional moiety shown in the box is linked to a sulfur atom of the disulfide substituent of a matrix M:



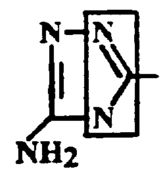
More specific X and Z structures in which the functional moiety shown in the box is linked to a sulfur atom of the matrix disulfide substituent are:



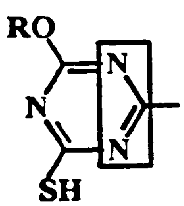
4-AMINOPYRIMIDINE GROUP



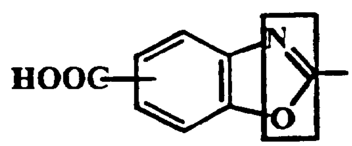
2-AMINO-1,3,4-THIADIAZOLE GROUP



3-AMINO-1,2,4-TRIAZOLE GROUP

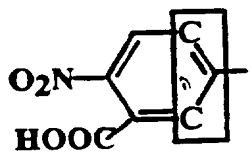


4-MERCAPTO-6-ALKOXYTRIAZINE GROUP

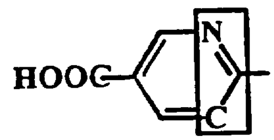


CARBOXYBENZOXAZOLE GROUP

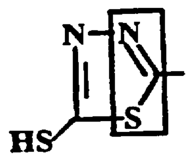
X and Z structures used in the ensuing examples and in which the disulfide sulfur linked functional moiety is shown in the box are:



2-NITROBENZOIC ACID GROUP

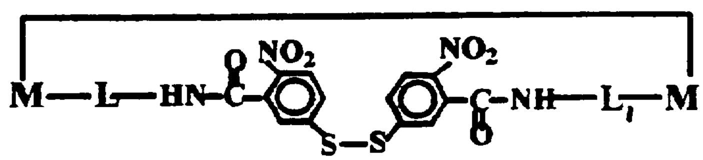


3-PYRIDINE CARBOXYLIC ACID GROUP



5-MERCAPTO-1,3,4-THIADIAZOLE GROUP

A preferred disulfide substituted resin of Formula II has the formula:



FORMULA III

The best mode presently known for producing the disulfide of Formula III is to couple M-L-NH_2 with 5-chloro-2-nitrobenzoic acid, followed by nucleophilic displacement of the aryl chloro group with the sulfur moiety of thiolacetic acid. The acetyl group on the resulting aryl sulfur moiety is hydrolytically removed by treatment with aqueous sodium carbonate. The Formula III resin disulfide product is then formed by air oxidation of the resin aryl mercapto groups, as described in the examples.

Because the disulfide substituent is doubly attached, contact of the resin with a thiol in solution will result in conversion of the disulfide to thione moieties without release of by-products.

Additionally, the invention provides for a dilution effect on the solution-phase thiol contacting the resin. This occurs as a result of the reaction, for example, of a monothiol reactant with resin-bound, widely spaced disulfide substituents

on the surface of the resin, thereby forming the intermediate mixed disulfide at a relatively low surface density. The decreased apparent concentration of resin-bound mixed disulfide leads to a slower reaction with a second monothiol molecule in solution to form the soluble dimeric disulfide product.

The dilution effect is especially useful for compounds or peptides containing two thiol groups per molecule where formation of an intramolecular disulfide bond is desired. Because it is bound to the resin (see Figure 5), the dithiol is effectively diluted on the resin surface (the so-called "pseudo dilution" effect). Consequently, the likelihood that the desired intramolecular disulfide will form is increased because the probability is decreased that a productive collision with a solution-phase dithiol molecule will occur to form an undesired dimeric molecule with an intermolecular disulfide bond. The result is that, in comparison with air oxidation in solution, a higher concentration of a peptidedithiol can be contacted with the resin to form the desired intramolecular disulfide bond without generating a significant amount of the undesired intermolecular disulfide-bonded peptide dimer.

It may be apparent that a novel and useful group of resins has been described for formation of disulfide bonds within and between molecules. Therefore, one object of the present invention is to provide solid support resins for synthesis of symmetrical intermolecular disulfides from thiols by a thiol-disulfide interchange reaction which is generally applicable to all thiols.

It is another object of the present invention is to provide solid support resins for synthesis of intramolecular disulfide bonds in dithiol or oligothiol precursors with two or more thiol groups such as, for example, peptidedithiols.

Another object of the present invention is to provide solid support resins for synthesis of intramolecular disulfide bonds in di- or oligothiol compounds at relatively high

concentrations compared with the corresponding reaction taking place wholly in solution. A higher concentration of soluble reactant can be employed in the present invention because of the "pseudo dilution" effect arising when solution-phase components react with substituents present at low surface density on a solid surface.

Yet another object of the present invention is to provide solid support resins which permit easy separation of soluble disulfide product from insoluble, resin-bound thiol product. These products are generated during the thiol-disulfide interchange reaction between a soluble thiol precursor and the disulfide substituent which is doubly linked to the resins via the moiety attached to each sulfur atom of the dithio group of the disulfide.

Yet another object of the present invention is to provide solid support resins which permit a nearly complete, i.e., at least 90% complete, conversion of soluble thiol to the soluble disulfide product by thiol-disulfide interchange. This is effected by employing resin-bound disulfide substituents which, when converted to their corresponding thiols during reversible thiol-disulfide interchange, undergo tautomerization to the corresponding thione derivatives. The formation of thiones thus effectively drives the interchange reaction to completion, rendering it essentially irreversible.

Lastly, another object of the present invention is to provide solid support resins which can generate soluble disulfides from soluble thiols by thiol-disulfide interchange not only in neutral and basic aqueous solutions, but also in acidic aqueous solutions in which conversion of thiols to disulfides generally proceeds slowly.

As the specification continues, it will be apparent that the present invention possesses other objects and advantages, especially those pertaining to particular characteristics and features. For example, the resins described herein could be

used to capture, isolate and recover monothiols under gentle conditions. They also could be used to prepare unsymmetrical disulfides.

ABBREVIATIONS

Abbreviation	Chemical name
Ac_2O	acetic anhydride
Boc	tert-butyloxycarbonyl
CDI	carbonyl diimidazole
DCM	dichloromethane
DIEA	diisopropylethylamine
DIPCDI	diisopropylcarbodiimide
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DMT	2,5-dimercapto-1,3,4-thiadiazole
DTDNA	6,6'-dithiodinicotinic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
DTTG	2,2'-dithiobis(1,3,4-thiadiazole-5-thioglycolic acid)
EDA	ethylene diamine
Fmoc	fluorenylmethyloxycarbonyl
HOBT	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
MNA	6-mercaptonicotinic acid
MNB	5-mercapto-2-nitrobenzoic acid
NHS	N-hydroxysuccinimide
NMP	1-methyl-2-pyrrolidinone
Ox	oxytocin
Ox-Ox	oxytocin dimer
OxH_2	dihydrooxytocin
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography

DEFINITIONS

Solid Support Resin: A normally solid synthetic resin matrix having a disulfide moiety linked covalently thereto, either directly or by a linker.

Matrix: The normally solid synthetic resin matrix of a solid support.

Jeffamine EDR-192: Tetraethylene Glycol Diamine - $H_2NCH_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$ (Texas Chemical Co., 3040 Post Oak Boulevard, Post Office Box 27707, Houston, Texas 77227-7707).

Jeffamine EDR-148: Triethylene Glycol Diamine - $H_2NCH_2CH_2OCH_2CH_2OCH_2NH_2$.

Jeffamine T-403: 1,1,1-tris(2-(2-aminopropyloxy)propyloxymethyl)propane plus 1,1-bis(2-2-aminopropyloxy)propyloxymethyl)-1-(2-aminopropyloxymethyl)propane. See Figure 6.

Polyhema: Hydroxyethyl methacrylate-ethylene dimethacrylate copolymer. See Figure 7. (Melcor Technologies, Inc., 1016 El Camino Real, Suite 435, Sunnyvale, California 04087).

Macroprep CM: Methacrylic acid/hydroxypropyl methacrylate-ethylene dimethacrylate copolymer. See Figure 8. (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California 94647).

Styrene-divinylbenzene (DVB) Copolymer: (1% divinylbenzene) commonly called 1% DVB-crosslinked polystyrene. See Figure 9. Novasyn TG resin and ω-aminopolyethylene glycol: Peg,n=@70) chains grafted onto styrene-divinyl benzene copolymer (1% divinylbenzene). See Figure 10. (Calbiochem-Novabiochem Corp.)

Aminomethylated 1% DVB-crosslinked polystyrene: 0.56 millimoles NH_2 per gram. See Figure 11.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention encompasses several different solid phase matrices each of which is attached to any one of several different linkers, L, which, in turn, is attached to any one of several disulfide substituents, -X-S-S-Z. A type of matrix, linker and disulfide substituent for each of solid support disulfide resins Nos. 1-8 (see Figures 12-19) described below is listed in the Table I.

TABLE I

Components of the preferred embodiments of the invention

Disulfide Resin No.	Matrix	Linker	Substituent
1	Polyhema	Jeffamine T-403	DTNB
2	Polyhema	Jeffamine EDR-148	DTNB
3	Polyhema	Ethylene diamine	DTNB
4	Polyhema	Jeffamine EDR-148	DTTG
5	Macro-Prep CM	Lysyl-Jeffamine EDR-192	DTNB
6	NovaSyn TG	Lysine	DTNB
7	S-DVB	Lysine	DTNB
8	S-DVB	Lysine	DTDNA

Examples I to VIII describe eight resins embodying various aspects of this invention.

EXAMPLE I

The first resin has a 5,5'-dithiobis (2-nitrobenzoic acid) substituent (DTNB) attached by each of its carboxyl groups via amide linkage to the distal amino groups of trifunctional Jeffamine T-403 (a polypropylene oxide-based triamine) which in turn, is attached by the remaining proximal amino group via urethane linkage to a hydroxyl group of 2-hydroxyethyl methacrylate-ethylene dimethacrylate copolymer (Polyhema). This resin is useful in preparing intramolecular disulfide bonds in neutral to mildly basic aqueous solutions. For example, peptidedithiols can be converted to the corresponding intramolecular peptide disulfides without contaminating the peptide solution with the dithio component and its reduced thiol moieties (see preceding diagram). The trifunctional Jeffamine component allows for very high dilution of DTNB groups on the resin surface by permitting DTNB to be crosslinked only intramolecularly to the two distal amino groups of a single Jeffamine molecule. Since the DTNB groups attached to the surface of this resin may be spaced far apart, a soluble protein molecule colliding with the resin surface will on average, access only one DTNB group. This wider

spacing of DTNB (low surface density) favors formation of intramolecular disulfide bonds in larger peptides or proteins containing two or more thiol groups rather than formation of intermolecular disulfide bonds as may be obtained using resins with a higher surface density of DTNB groups.

Resin # 1 may be prepared by the sequence of 5 reactions as depicted by Figure 12. The R_1 enclosed within a circle in the Figure 12 diagram indicates 2-hydroxyethyl methacrylate-ethylene dimethacrylate copolymer (Polyhema) with a rigid, macroporous structure. r, S, and t subscripts in the Jeffamine T-403 structure represent the moles of propylene oxide units such that r+S+t = 5-6 moles per mole Jeffamine. In the figure, -S... indicates a crosslink to a sulfur atom of a disulfide moiety of a like formula.

The production and analysis of one embodiment of Resin #1 is illustrated by the following experiment.

5.0 gm of 2-hydroxyethyl methacrylate-ethylene dimethacrylate copolymer (Polyhema, 2.2 mmoles of hydroxyl groups per gram of resin) was suspended in 30 ml of dimethylformamide (DMF). 3.6 gm of carbonyldiimidazole (CDI) was dissolved in 50 ml of a 1:1 (volume:volume) mixture of dry DMF and dichloromethane (DCM). This solution was added to the Polyhema suspension, and the resultant suspension was mixed overnight at room temperature. The resin was collected by filtration and washed three times with DMF. The resin cake was resuspended in 50 ml of Jeffamine T-403 (1,1,1-tris(hydroxy-methyl)propane in ether linkage with three ω-aminopolyoxypropylene chains), and the suspension was mixed overnight at room temperature. 100 ml of DMF was added to the suspension, and after mixing for 1 hour at room temperature, the resin was collected by filtration and washed once with methanol, once with DMF and twice with DCM. After air drying, the resulting yield of Jeffamine resin was 7.52 gm. Nitrogen

elemental analysis of a sample of the dried resin gave 0.97 mmoles Jeffamine T-403 per gm of resin, from which result it was calculated that 80% of the available hydroxyl groups on the Polyhema resin had been derivatized. 1.74 gm of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was dissolved in 10 ml of DMF, and 1.4 ml of diisopropylcarbodiimide (DIPCDI) was added. After ten minutes, 1.5 gm of Jeffamine resin was added, and the suspension was mixed overnight at room temperature. The resin was collected by filtration, washed twice with DMF and twice with DCM and air dried. A sample of the dried resin was tested with ninhydrin for the presence of primary amino groups (purple color upon heating with ninhydrin in acidic butanol). 1.74 gm of DTNB was dissolved in 10 ml of DMF containing 1.4 ml of DIPCDI. This solution was added to the resin, and the suspension was mixed overnight at room temperature. The resin was collected by filtration and washed twice with DMF and twice with DCM. The residual amino groups on the resin were acetylated by adding the resin to 10 ml of acetic anhydride (Ac_2O). The suspension was mixed for 1 hour at room temperature, and the resin was collected by filtration, washed twice with DMF and twice with DCM and air dried. A sample of the dried resin gave a negative result in the ninhydrin test for the presence of primary amine groups. The resin-bound DTNB groups were reduced by suspending the dried DTNB resin in 10 ml of DMF and adding 1.4 gm of dithiothreitol (DTT). The suspension was mixed for two hours at room temperature. The resulting 5-mercapto-2-nitrobenzoyl (MNB) resin was collected by filtration, washed four times with DMF and twice with DCM and air dried. The dry, red MNB resin was resuspended in DMF and reoxidized to pale yellow DTNB resin by bubbling air through the suspension overnight at room temperature. The reoxidized DTNB resin was collected by filtration, washed three times with DMF and three times with DCM and air dried. Treatment of a sample of the dry resin with

a solution of DTT in DMF caused the resin to turn red immediately (reduction of DTNB to MNB). Elemental analysis of a sample of the resin revealed a nitrogen substitution of 3.03 millimoles per gm of resin and a sulfur substitution of 0.63 millimoles per gm resin. Since DTNB has two sulfur atoms per mole, the DTNB substitution of the reoxidized resin was 0.32 millimoles per gm resin. From the excess nitrogen, it was calculated that the Jeffamine T-403 substitution was 0.8 millimoles per gm resin, which represents a 71% conversion of the hydroxyl groups of the Polyhema starting material (2.2 millimoles OH groups per gm resin).

The DTNB resin product was examined for its capacity to convert reduced oxytocin (OxH_2) to oxytocin containing an intramolecular disulfide bond (Ox). Authentic OxH_2 was dissolved at 1 mg/ml or 10 mg/ml in 50 volume % of aqueous acetonitrile. One 4 mg and one 30 mg sample of DTNB resin (1.3 and 9.6 micromoles of resin-bound DTNB, respectively) were mixed with 1 ml of 50% acetonitrile. The suspensions were centrifuged, and the supernatants were removed and discarded. 5 μl of the 10 mg/ml OxH_2 solution was added to the wetted 4 mg sample every 5 minutes with stirring after addition until 25 μl (0.25 millimoles of OxH_2) was added over a 30 minute period. At this point, a 1 μl aliquot of the resin suspension supernatant was spotted onto a silica gel TLC plate. The addition every five minutes of a 5 μl aliquot to the 4 mg resin sample was continued until another 25 μl had been added at the end of a second 30 minute interval for a total addition of 500 μg (0.5 millimoles) of OxH_2 over 60 minutes. A 1 μl aliquot of this final resin suspension supernatant was also spotted on the TLC plate. 500 μl of the 1 mg/ml OxH_2 solution was added to the 30 mg DTNB resin sample. The suspension was mixed for 60 minutes and centrifuged, and the supernatant was recovered and lyophilized. A small amount of the lyophilized residue was dissolved in 50% aqueous acetonitrile, and a 1 μl aliquot was

applied onto the TLC plate along with OxH_2 and Ox standards. The TLC plate was dried and developed with an ascending mobile phase consisting of butanol:pyridine:acetic acid:water in the volume ratio, 15:10:3:12. After development, the plate was dried and visualized by exposure to iodine vapor. A photograph of the TLC chromatogram is included as Figure 20. Table II shows the results of this analysis.

TABLE II

Oxidation of reduced oxytocin (OxH_2) with DTNB resin (Resin # 1)

#	Sample	Resin (mg)	Time (minutes)	OxH_2: DTNB	TLC R_f
1	OxH_2 standard	0	0	----	0.80
2	Ox standard	0	0	----	0.58
3	OxH_2+resin	4	30	1:5.2	0.56
4	OxH_2+resin	4	60	1:2.6	0.56
5	OxH_2 standard	0	60	----	0.76
6	OxH_2 standard	0	60	----	0.76
7	Ox standard	0	60	----	0.54
8	OxH_2+resin	30	60	1:19.5	0.54

All oxidations of reduced oxytocin with DTNB resin were complete inasmuch as no TLC spot at the R_f of OxH_2 was observed in the resin supernatants.

Sample #'s 1, 2, 4 and 8 in Table II were lyophilized, reconstituted in 0.1 % trifluoroacetic acid (TFA) and examined by analytical, gradient-elution reverse phase HPLC. Elution was effected with a gradient of 0% Solvent A (0.1% TFA in water) to 60% solvent B (0.09% TFA in acetonitrile) over 30 minutes, and eluted peptides were detected by 214 nm absorption. The HPLC elution profiles are shown in Figure 21. Table III below is a display of the HPLC peak data (area and retention time) for HPLC of sample #'s 1, 2, 4 and 8 from Table II.

TABLE III

HPLC of Ox, OxH_2, and products of OxH2 oxidation with DTNB resin (Resin #1)

#	Sample	Retention Time (min)			Area			%Area		
		Ox	OxH_2	Ox-Ox	Ox	OxH_2	Ox-Ox	Ox	OxH_2	Ox-Ox
1	OxH_2	16.3	16.6	-----	191	7141	-----	2.6	97	0
2	Ox	16.3	16.6	-----	6274	245	-----	96	3.8	0
4	OxH_2+ Resin	16.3	16.5	17.6	2331	51	787	74	1.6	25
8	OxH_2+ Resin	16.3	16.5	17.6	3861	97	256	92	2.3	6.1

Mass spectroscopic analysis of the major peaks in the HPLC eluate of Sample # 4 (see Table IV) revealed that the peak eluting at 17.6 minutes had a mass corresponding to Ox-Ox which represents oxytocin dimer arising from formation of two intermolecular disulfide bonds per dimer. Sample # 4 with a resin DTNB-to-OxH_2 molar ratio of 19:1 produced much less Ox-Ox than did Sample # 4 which had a DTNB:OxH_2 ratio of 2.6:1. Therefore, intramolecular disulfide bond formation requires use of a molar excess of resin-bound DTNB over dithiol component.

The HPLC peaks for Sample #4 in Table III were collected and submitted for mass spectrometric analysis, the spectrograms of which are shown in Figures 22, 23 and 24. The results of this study are shown in Table IV.

TABLE IV

Mass spectrometry of the products of OxH_2 oxidation with DTNB resin (Resin # 1)

Peptide	Retention time (min)	Molecular weight	Mass by Analysis
Ox	16.3	1007	------
OxH_2	16.6	1009	------
Ox-Ox	-------	2014	------
peak # 1	16.3	-------	1007
peak # 2	17.2	-------	1561
peak # 3	17.6	-------	2014

Peak # 1 is Ox and Peak # 3 is Ox-Ox with an HPLC retention time of 17.6 minutes. Peak # 2 does not correspond to a known form of oxytocin and may represent a truncated peptide contaminant arising during synthesis of oxytocin.

EXAMPLE II

Resin #2 also has a DTNB substituent attached by both carboxyl groups to Polyhema resin. The DTNB is crosslinked by intermolecular amide bonds to the distal amino group of each of two bifunctional Jeffamine EDR-148 molecules (triethylene glycol diamine) each of which is attached to the resin via a urethane linkage between the proximal amino group and a resin hydroxyl group. A resin with a higher density of DTNB groups on the support may be used to convert smaller peptidedithiols to the corresponding intra-molecular peptide disulfides.

Resin #2 may be prepared as in the following sequence of 5 reactions as depicted in Figure 13. R_1 is Polyhema resin and Jeffamine EDR-148 is triethylene glycol diamine.

The production and analysis of one embodiment of Resin #2 is illustrated by the following experiment.

50 gm of Polyhema (see example 1) was suspended in 200 ml of DMF. 40 gm of CDI was dissolved in 300 ml of DMF, and the resultant solution was added to the resin suspension. The suspension was mixed by argon sparging overnight at room temperature. The resin was filtered and washed 3 times with 250 ml of DMF. 200 ml of triethylene glycol diamine (Jeffamine EDR-148) was added to the resin, and the suspension was mixed by argon sparging overnight at room temperature. The Jeffamine resin was filtered and washed 3 times with @ 200 ml of DMF. 65 gm of DTNB and 42 gm of N-hydroxysuccinimide (NHS) were dissolved in 500 ml of DMF, and 57 ml of diisopropylcarbodiimide (DIPCDI) and 500 ml of dimethylsulfoxide (DMSO) were added. The solution was stirred 4 hours at room temperature and then added to the suspension of Jeffamine resin. The suspension was mixed by argon sparging

for 48 hours at 50°. The orange resin was filtered and washed 3 times with DMF, 3 times with methanol, 3 times with DCM and twice with pentane. The resin cake was air dried for 15 minutes and then vacuum dried for 4 hours. 34.5 gm of DTNB was dissolved in 400 ml of DMSO, 15 ml of DIPCDI was added and the solution was mixed at room temperature for 30 minutes. The dried DTNB resin was added to this solution to recouple DTNB to residual amino groups on the resin, and the suspension was mixed for 2 hours at room temperature. 20 ml of diisopropylethylamine (DIEA) was added, and the suspension was left overnight at room temperature. The supernatant over the settled resin was decanted and 100 ml of acetic anhydride was added to acetylate any remaining amino groups. The suspension was heated at 50° for 3 hours and then filtered, washed 3 times with DCM and 3 times with methanol and air dried. A sample of the resin gave a negative ninhydrin test for primary amines. 75 gm of dithiothreitol was dissolved in 300 ml of DMF, 25 ml of N-methylmorpholine was added and the solution was mixed by swirling. The dried DTNB resin was added, the suspension was swirled and then left standing overnight at room temperature to effect reduction of the resin-bound DTNB groups. The DTNB resin was filtered and washed 3 times with alternating washes of DMF and methanol, twice with alternating washes of DCM and methanol, three times with DCM and twice with pentane. The washed resin was air dried and then vacuum dried overnight. The resin was resuspended in 250 ml of DMF, and filtered air was drawn through the suspension for 72 hours at room temperature to effect reoxidation of resin-bound 5-mercapto-2-nitrobenzoyl moieties to DTNB groups. The resin was filtered and washed twice with DCM, twice with alternating washes of methanol and DCM, three times with DCM, and three times with pentane. The resin cake was air dried for 30 minutes and vacuum dried overnight. 75 gm of light yellow resin was obtained. Elemental analysis of a sample of the

dried resin revealed a nitrogen content of 3.43 millimoles per gm of resin and a sulfur content of 0.71 millimoles per gm of resin. Since DTNB has two sulfur atoms per molecule, the DTNB content of the reoxidized resin was 0.36 millimoles per gm of resin. From the nitrogen in excess of that present in DTNB, it was calculated that the resin contained 1.36 millimoles of Jeffamine EDR-148 groups per gm of resin, which represents a 97% conversion of the hydroxyl groups of the Polyhema starting material.

The oxidation of reduced oxytocin with this DTNB resin (Resin #2) was examined at different molar ratios of OxH_2:DTNB. A varied amount of Resin # 2 was placed in small vials, and 75 μl of 50% aqueous acetonitrile was added. 200μl of a 1 mg/ml solution of OxH_2 in 50% aqueous acetonitrile was added to each vial, and the resultant suspensions were mixed for 4 hours at room temperature. The vials were centrifuged, and the supernatants were each transferred to a vial and lyophilized. The dried samples, along with authentic Ox and OxH_2, were submitted for HPLC analysis the chromatograms of which are included in Figure 25. The area % for Ox, OxH_2 and Ox-Ox peaks in the HPLC chromatograms are shown in Table V below.

TABLE V

HPLC of the products of OxH_2
Oxidation with CTNB resin (Resin # 2)

Sample	Resin Weight (mg)	OxH_2:DTNB	% of combined area Ox	OxH_2	Ox-Ox
OxH_2 stndrd	-----	-----	3.6	88	8.4
OxH_2+resin	6	1.10	92	4.7	3.8
OxH_2+resin	12	1.20	90	5.0	5.2
OxH_2+resin	48	1.80	89	4.9	5.8

The kinetics of conversion of OxH_2 to Ox using Resin # 2 were examined using Ellman's reagent to measure the concentration of remaining thiol groups. 12 mg of Resin #2

(4.3 micromoles of DTNB groups) was placed in each of 6 Eppendorf tubes. 20 ìl of 50% aqueous acetonitrile was added to each, followed by addition of 200 ìl of a 1 mg/ml solution of OxH_2 (0.2 micromoles) in 50% aqueous acetonitrile . The suspensions were mixed at room temperature and at each time point, a tube was centrifuged and the supernatant was recovered for analysis of thiol groups by reaction with Ellman's reagent. The latter was prepared by dissolving DTNB at 4 mg/ml in 0.1 M phosphate buffer, pH 8. Thiol group concentration was determined in duplicate by adding 30 μl of supernatant from the resin suspension to 1 ml of 0.1 M phosphate buffer, pH 8, followed by addition of 60 μl of Ellman's reagent. The resultant solution was mixed and, after 15 min, the absorbence at 412 nm was measured spectrophotometrically against a blank prepared by adding 30 μl of 50% acetonitrile and 60 μl of Ellman's reagent to 1 ml of the phosphate buffer. The concentration of peptide in each supernatant was likewise determined spectrophotometrically by diluting 40 μl of supernatant in 1 ml of 50% aqueous acetonitrile and measuring the absorbence at 214 nm against a blank of 50% aqueous acetonitrile. The results of this study are shown in Table VI below.

TABLE VI

OxH_2 thiol and peptide remaining after reaction with DTNB resin (Resin # 2)

Reaction time (min)	Thiol concentration (Absorbence at 412 nm)	Peptide concentration (Absorbence at 214 nm)
0	0.598±0.014	0.541
15	0.025±0.002	0.549±0.006
30	0.009±0.000	0.533±0.011
60	0.006±0.000	0.547±0.004
120	0.004±0.000	0.551±0.002
240	0.003±0.001	0.553±0.002
1080	0.001±0.000	0.538±0.004

The results in Table VI indicate that an insignificant amount of peptide becomes bound to the resin during the thiol-disulfide interchange reaction (from the 214 nm absorbence data), suggesting that formation of the intramolecular disulfide bond in oxytocin occurs rapidly with respect to initial formation of the resin-bound mixed disulfide precursor.

To determine if acetonitrile influences the conversion of OxH_2 to Ox by DTNB groups on Resin # 2, the reaction in 50% aqueous acetonitrile was compared with the reaction in water. 200 μl of a 1 mg/ml solution of OxH_2 in argon-sparged water was added to each of two tubes, one containing 12 mg of Resin # 2 and 200 μl of acetonitrile and the other containing 12 mg of Resin #2 and 200 μl of argon-sparged water. The suspensions were mixed at room temperature for 2 hours, and 1 μl of TFA was added to each vial to slow air oxidation of thiol groups. Each sample was submitted for HPLC analysis as described above. The results showed no difference in DTNB resin-induced formation of Ox from OxH_2 in 50% aqueous acetonitrile versus water.

To determine if oxytocin is adsorbed onto the DTNB resin, suspensions containing Ox and an increasing volume % of acetonitrile were examined spectrophotometrically. 12 mg of Resin # 2 was placed in each of 4 tubes containing 400 μl of a solution with a varied amount of acetonitrile. The tubes were mixed for 2 hours at room temperature, and 50 μl of supernatant from each tube was diluted into 5 ml of distilled water. The 214 nm absorbency of each solution was measured, and the results are shown in Table VII.

TABLE VII

Adsorption of peptide to DTNB resin (Resin # 2)

% Acetonitrile	Resin (mg)	214 nm Absorbence	% Recovery
0	0	0.170	------
0	12	0.160	94
10	12	0.169	99
20	12	0.174	102
40	12	0.193	114

The results in Table VII show that peptide in purely aqueous solution is only slightly adsorbed to the resin, and that 10% acetonitrile totally prevents the adsorption.

EXAMPLE III

Resin #3 is identical to Resin # 2 except that the bifunctional Jeffamine EDR-148 linker is replaced by the shorter bifunctional spacer, ethylene diamine.

Resin #3 is prepared using Polyhema resin as depicted in Figure 14.

The production and analysis of one embodiment of Resin #3 is illustrated by the following experiment.

891 mg of CDI was dissolved in 10 ml DMF, and the solution was added to 1 gm of Polyhema resin. The suspension was mixed 3 days at room temperature. The resin product was filtered and washed with DMF, and a sample was removed, washed twice with DCM, dried and submitted for nitrogen elemental analysis. The analysis revealed a nitrogen content of 2.06 millimoles per gm of resin, which represents a 94% conversion of Polyhema hydroxyl groups to the oxycarbonyl imidazolide derivative. The remainder of the resin was added to 10 ml of ethylenediamine (EDA), and the suspension was mixed overnight at room temperature. 20 ml of DMF was added, and the resin was mixed an additional 30 minutes. The EDA resin was filtered, washed with DMF until the filtrate was negative for primary amino groups by the ninhydrin test. The resin was washed further with alternate washes of DMF and DCM, once more with DCM and

then air dried and vacuum dried. The yield of EDA resin was 1.2 gm (106% of theory). 1.74 gm of DTNB was dissolved in 10 ml of DMF, 1.4 ml of DIPCDI was added, and the solution was mixed for 5 minutes at room temperature. The solution of activated DTNB was then added to the dried EDA resin, and the suspension was mixed overnight at room temperature. The resultant DTNB resin was filtered, washed alternately with DMF and DCM and then with DCM. Residual amino groups were acetylated by suspending the resin in 10 ml of acetic anhydride and mixing for 3 hours at room temperature. The resultant "capped" resin was filtered, washed alternately with DMF and DCM, followed by DCM alone and air dried. A ninhydrin test of a sample of the dried capped DTNB resin was negative for amino groups. The DTNB resin was resuspended in 8.8 ml of 0.5 M $NaBH_4$ in diglyme. After 1 hour at room temperature, 10 ml of DMF was added and the red suspension was mixed overnight at room temperature. The red-orange resin was filtered, washed with DMF until the filtrate was colorless and resuspended in 50 ml of 50% acetic acid. After mixing for 15 minutes, the resin was filtered, washed twice with DMF, and resuspended in 20 ml of DMF containing 200 μl of DIEA. Air was drawn through the suspension overnight at room temperature to effect oxidative crosslinking of the resin-bound 5-mercapto-2-nitrobenzoyl groups to form DTNB groups. The pale yellow DTNB resin was filtered, washed twice alternately with DMF and DCM, then DCM and air dried. The resin (1.2 gm) was dried in vacuuo. Nitrogen and sulfur analysis of a sample of the dried DTNB resin revealed a nitrogen substitution of 4.56 millimoles per gm of resin, and a sulfur substitution of 0.29 millimoles per gm of resin. Since DTNB contains two sulfur atoms per mole, the sulfur substitution translates into a DTNB substitution of 0.15 millimoles per gm of resin. The excess nitrogen not associated with DTNB groups gives an EDA substitution of 2.1 millimoles per gm of resin which represents 100% conversion of

the oxycarbonylimidazolide groups of CDI-reacted Polyhema to EDA groups. Treatment of a sample of the resin with DTT in DMF results in a change of resin color from pale yellow to red, indicating reduction of the DTNB group to the red thione form of the 5-mercapto-2-nitrobenzoyl group. Since this resin is similar to Resin # 2 except for substitution of ethylene diamine for the triethylene glycol diamine linker, no further studies were conducted with Resin # 3.

EXAMPLE IV

Resin #4 is identical to Resin #2 except that the DTNB substituent has been replaced by a 2,2'-dithiobis(1,3,4-thiadiazole-5-thioglycolic acid) substituent (DTTG) which, unlike the DTNB resins, is useful for converting thiols to disulfides in acidic solutions.

Resin # 4 is prepared by the sequence of 5 reactions as depicted in Figure 15. R_1 is Polyhema, DMT is 2,5-dimercapto-1,3,4-thiadiazole and Jeffamine EDR-148 is triethylene glycol diamine.

The production and analysis of one embodiment of Resin #4 is illustrated by the following experiment.

45 gm of Polyhema resin and 40 gm of CDI were suspended in 300 ml DMF, and the suspension was mixed by sparging with argon overnight at room temperature. The resin was filtered and washed three times with @ 250 ml of DMF. 200 ml of triethylene glycol diamine (Jeffamine EDR-148, Texaco) was added to the resin, and the suspension was mixed by argon sparging overnight at room temperature. The resultant Jeffamine resin was filtered and washed three times with DMF, three times with DCM, twice with alternate double washes of pentane and DCM and then three times with pentane. The resin was air dried and then vacuum dried for 2 hours. A sample submitted for nitrogen elemental analysis revealed a Jeffamine substitution of 1.44 millimoles per gm of resin (87% of theory). 5.6 gm of bromoacetic acid was dissolved in 15 ml of DMF, and 3.1 ml of

DIPCDI was added. The solution was mixed for 15 minutes at room temperature and then added to a suspension of 5 gm of Jeffamine resin in 15 ml of DMF. The resin suspension was mixed for 2 hours at room temperature. The resulting bromoacetamido resin was filtered, washed five times with alternating double washes of DCM and pentane and twice with pentane and then air and vacuum dried. A sample of the dried bromoacetamido resin gave a negative result in the ninhydrin test for primary amino groups. 1 gm of 2,5-dimercapto-1,3,4-thiadiazole was dissolved in 2 ml of DMF and 350 μl of DIEA was added. After mixing for 2 minutes, this solution was added to a suspension of 1 gm of bromoacetamido resin in 2 ml of DMF. The suspension was heated overnight at 50°. The resin was filtered, washed and dried as above for the bromoacetamido resin. A sample of the 5-mercapto-1,3,4-thiadiazole-2-thioglycolamido resin product was submitted for nitrogen and sulfur elemental analysis which revealed a nitrogen content of 4.04 millimoles per gm of resin and a sulfur content of 2.41 millimoles per gm of resin. From these results, it was calculated that the resin content of 5-mercapto-1,3,4-thiadiazole-2-thioglycolyl groups was 0.8 millimoles per gm of resin (66% of theory). The dried resin was suspended in DMSO, and air was drawn through the suspension for 24 hours at room temperature to oxidize the resin thiol substituents to dithio groups, yielding the 2,2'-dithiobis(1,3,4-thiadiazole-5-thioglycolyl)-substituted resin (DTTG resin) at 0.4 millimoles DTTG per gm.

The efficacy of this resin in converting thiols to disulfides under acidic aqueous conditions was examined using reduced oxytocin (OxH_2). Ellman's reagent for spectrophotometric determination of thiol groups was applied to measure OxH_2 thiol groups remaining after a varied time of exposure to the DTTG resin. 500 μl of a 1 mg/ml solution of OxH_2 in 50% aqueous acetonitrile containing 0.1% TFA was added

to 20 mg of DTTG resin to give a DTTG:OxH_2 molar ratio of 16:1. At each time point (1, 2, 4, 8, 16, 60 and 120 minutes), 20 μl of supernatant was transferred to a tube. After the last time point, 150 μl of a 4 mg/ml solution of DTNB in 0.1 M phosphate buffer, pH 8, was added to each tube. After mixing, 2 ml of 0.1 M phosphate buffer, pH 8, was added, and the tubes were again mixed. After 15 minutes, the absorbence at 412 nm was measured. A zero time point solution was prepared by adding 20 μl of OxH_2 solution and 150 μl of DTNB solution to 2 ml of the phosphate buffer. The results are presented in Table VIII below. The 412 nm reading for the two hour supernatant was taken as the blank, since a negligible reduction in absorbence occurred after 60 minutes.

TABLE VIII

OxH_2 thiol groups remaining after reaction with DTTG resin (Resin #4)

Reaction time (min)	Thiol concentration (Absorbence at 412 nm)	% Thiol groups remaining
0	0.199	100
1	0.179	90
2	0.143	72
4	0.101	51
8	0.076	38
16	0.058	29
60	0.002	1.0

Since oxidation of thiol groups in aqueous acids is relatively slow, the DTTG resin shows remarkable efficacy in converting OxH_2 to its dithio derivatives. To determine the extent of formation of Ox, the intramolecular disulfide form of oxytocin versus the intermolecular disulfide, Ox-Ox, the supernatant from the two hour reaction was submitted for reverse phase HPLC analysis by the gradient elution system described above for Resin # 1. Figure 26 shows the HPLC chromatograms corresponding to the results shown in Table IX below.

TABLE IX

HPLC of the products of OxH_2 oxidation with DTTG resin (Resin # 4) at acid pH

Sample	Retention Time (min)			% of combined area		
	Ox	OxH_2	Ox-Ox	Ox	OxH_2	Ox-Ox
OxH_2 standard	16.2	16.5	17.5	2.5	92	5.3
OxH_2+resin (2 hrs)	16.4	16.7	17.7	84	1	15

The HPLC results are concordant with the results in Table VIII showing nearly complete conversion of OxH_2 to largely Ox plus some Ox-Ox dimer.

EXAMPLE V

Resin # 5 has a DTNB substituent with both carboxyl groups attached in amide linkage to the α and ε amino groups of lysine moieties which are amide-bonded through the lysine carboxyl group to the distal amino group of a bifunctional Jeffamine EDR-192 molecule (tetraethylene glycol diamine). The latter is linked by an amide bond between its proximal amino group and a craboxyl group on a hydrophilic methacrylate-ethylene methacrylate copolymer (Macro-Prep CM support, BIO-RAD). This resin is similar to Resin #1 in its applications, since the resin may be prepared such that the DTNB-lysine substituents are present at low surface density on the resin. As in Resin #1, this favors formation of intramolecular disulfide bonds in larger peptides and proteins containing two or more thiol groups.

Resin # 5 is prepared by the sequence of reactions depicted in Figure 16. R_2 in the Figure 16 diagram is a hydrophilic, macroporous methacrylate copolymer (Macro-Prep CM), Boc is the butyloxycarbonyl protecting agent for amino groups and Jeffamine EDR-192 is tetraethylene glycol diamine. At a high surface density of lysine groups, the DTNB may crosslink two resin-bound lysines, whereas at low lysine density, DTNB is

mostly attached to the two amino groups on a single resin-bound lysine moiety.

The production and analysis of one embodiment of Resin #5 is illustrated by the following experiment.

16 gm of methacrylate-ethylene methacrylate copolymer (Macro-Prep CM, BioRad) was suspended in a solution prepared from 12 ml of tetrahydrofuran (THF), 8 ml of pentane and 20 ml of tetraethylene glycol diamine (Jeffamine EDR-192, Texaco). The suspension was mixed for 1 hour at room temperature to form the Jeffammonium resin-carboxylate salt and filtered, washed five times with a 60:40 mixture of THF:pentane, air dried and then vacuum dried. The dry resin was resuspended in 25 ml of DCM, 2.4 ml of DIPCDI was added, and the suspension was mixed overnight at room temperature. The resultant Jeffamine resin was filtered, washed successively with methanol, DCM, and pentane, then air dried for 15 minutes and vacuum dried overnight. 8.9 gm of N-α-N-ε-bisBocl-lysine and 4.1 gm of 1-hydroxybenzotriazole (HOBT) were dissolved in 160 ml of DMF. 4.2 ml of DIPCDI was added, and the solution was mixed for 15 minutes at room temperature. 16 gm of Jeffamine Macro-Prep-CM resin was added, and the suspension was mixed overnight at room temperature. The resin was filtered, washed twice with DMF, alternately with methanol and DMF, then twice with methanol and air dried. The dried resin was recoupled in 160 ml of DMF containing half the amount of bis-Boc-lysine, HOBT and DIPCDI used in the first coupling. The doubly coupled Boc-lysine resin was filtered, washed twice with DMF, alternately with methanol and DMF, twice with methanol and then air dried. The Boc protecting group was removed by suspending the resin in TFA:water, 95:5 (volume:volume) for 4 hours at room temperature. The resin was filtered, washed with DCM, then with 10% DIEA in DCM in order to convert the resin-bound trifluoroacetate salt of the lysine amino group to the free amine form. The lysine resin was washed with DCM and pentane,

air dried and then vacuum dried. 10 gm of DTNB and 6.1 gm of HOBT were dissolved in 120 ml of DMF. 6.3 ml of DIPCDI was added, and the suspension was mixed for 20 minutes at room temperature. 10 gm of lysine resin was added, and the suspension was mixed 24 hours at room temperature. The DTNB resin product was filtered, washed twice with DMF, alternately with methanol and DMF, and resuspended in @ 20 ml of acetic anhydride to acetylate or "cap" the remaining amino groups. After filtering and washing the resin, the DTNB substituent of the "capped" resin was reduced to the 5-mercapto-2-nitrobenzoyl group (MNB) by room temperature, 4-hour treatment with a 5-fold molar excess of DTT in DMF. The red MNB resin was filtered and washed, resuspended in DMF, and air was drawn through the suspension for 2 days to reoxidize resin-bound MNB groups to the DTNB substituent which was doubly linked to the resin. The pale yellow DTNB resin (Resin # 5) was filtered, washed three times with methanol, air dried and then vacuum dried overnight. Resin # 5 was examined for its capacity to convert OxH_2 to Ox by thiol-disulfide interchange. 2 mg of authentic OxH_2 was dissolved in 1 ml of argon-sparged water. For the 214 nm zero time point, 20 μl of this solution was diluted to 1 ml with degassed water, and the 214 nm absorbence of the resultant solution was measured. For the 412 nm zero time point, 20 μl of the OxH_2 solution was added to 1 ml of 0.2 M phosphate buffer, pH 7.8, to which 100 μl of DTNB reagent (4 mg/ml DTNB in 0.2 M phosphate buffer, pH 7.8) had been added, and 15 minutes thereafter, the 412 nm absorbence was measured. For the other time points (5, 20, 30, 60 and 120 minutes), 400 μl of the OxH_2 solution was added to 10 mg of Resin # 3, and the suspension was mixed at room temperature. At each time point, one 20 μl aliquot of supernatant was removed and diluted in 1 ml of water for 214 nm absorbence measurement. Another 20 μl aliquot was removed and diluted in 1 ml of 0.2 M phosphate buffer, pH 7.8. 100 μl of DTNB reagent was added, the solution

was mixed and 15 minutes thereafter, the 412 nm absorbence was measured. The results of this photometric assay for thiol groups remaining after a varied time of treatment with Resin # 6 are shown in Table X below.

TABLE X

OxH_2 thiol groups and OxH_2 remaining after reaction with DTNB resin (Resin # 5)

Reaction time (min)	Thiol concentration (412 absorbence)	Peptide concentration (214 absorbence)
0	0.541	0.475
5	0.264	0.465
20	0.185	0.480
30	0.164	0.470
60	0.075	0.450
120	0.030	0.438

At two hours, only 6% of the initial thiol groups remain, and no significant accumulation of peptide on the resin occurs, suggesting that formation of the intramolecular disulfide bond in Ox is rapid compared with formation of its resin-bound mixed disulfide precursor, MNB-S-S-Ox-SH.

EXAMPLE VI

Resin #6 has DTNB substituents attached by both carboxyl groups in amide linkage to the α and ε amino groups of resin lysine substituents. Lysine is amide-bonded via its carboxyl chain which is linked to a styrene-divinylbenzene copolymer (NOVASYN-TG, NovaBiochem). This resin is similar to Resin #1 and #5 in its applications involving larger peptides and proteins.

Resin #6 is prepared is prepared by the reactions depicted in Figure 17. R_3 in the Figure 17 diagram consists of ω-aminopolyoxyethylene chains grafted onto a crosslinked styrene-divinylbenzene copolymer.

The production, analysis and utility of Resin #6 is illustrated by the following experiment.

1 gm of NovaSyn TG resin, 336 mg of N-α-N-ε-bisFmoc-lysine and 92 mg of HOBT were added to 5 ml of DMF. After mixing to dissolve the nonresin components, 93 μl of DIPCDI was added, and the suspension was mixed overnight at room temperature. The bisFmoc-lysine resin was filtered, washed twice with DMF, twice with methanol, twice with DCM, once with methanol and twice with DCM and air dried. A sample of the resin gave a negative ninhydrin test for primary amino groups, indicating complete coupling of the resin amino groups. The Fmoc protecting groups were removed by suspending the bisFmoc-lysine resin in 10 ml of 20 volume % piperidine in DCM and mixing the suspension for 30 minutes at room temperature. The resultant lysine resin was filtered, washed twice with alternate DCM and methanol washes and then twice with methanol. The resin was air dried, and a sample of the resin gave a positive ninhydrin test result, indicating removal of the Fmoc protecting group. 475 mg of DTNB and 199 mg of HOBT were dissolved in 7 ml of DMF, and 203 μl of DIPCDI was added. After mixing for 15 min, the solution was added to the lysine resin, and the suspension was mixed overnight at room temperature. The DTNB resin was filtered and washed with DMF. A sample of the resin gave a negative result in the ninhydrin test. The resin was further washed, twice with alternating washes of methanol and DMF and twice with methanol, and then air dried. The DTNB resin was resuspended in 5 ml of DMF, 370 mg of DTT was added, and the red suspension was mixed for 2 hours at room temperature. The resultant MNB resin was filtered and washed with DMF until the filtrate was no longer reddish in color. The resin was then washed twice with DMF and twice with methanol and air dried. The dried resin was brick red in color, indicating the presence of MNB groups in the thione tautomeric form. A basic DMF solution was prepared by shaking 0.5 ml water and 5 gm NaOH with 50 ml DMF. 5 ml of the supernatant of this suspension was added to the MNB resin, and

the suspension was mixed for 10 minutes at room temperature. 254 mg of iodine was dissolved in 1 ml of the basic DMF solution, and 110 μl of the resultant solution was added to the resin suspension. The resin turned light yellow in color, indicating reoxidation of the resin-bound MNB groups to DTNB substituents. The resin was filtered and washed twice with DMF, twice with methanol and then twice with DMF. The slightly red resin was resuspended in DMF and 20 μl of the iodine solution was added. The resin again became pale yellow in color and remained so after washing twice with DMF and twice with methanol. The resin was dried, and a sample was treated with DMF containing DTT. The pale yellow resin (Resin # 6) turned dark brick red with no color appearing in the supernatant, indicating that the DTNB substituents were doubly linked to the resin and thus released no MNB upon reduction with DTT.

Resin # 6 was examined for its capacity to convert OxH_2 to Ox. 1.6 mg of OxH_2 was dissolved in 200 ml of degassed water, and 100 ml of this solution was mixed with each of two 5 mg samples of Resin # 6. After 5 minutes, an aliquot of supernatant was removed and, along with authentic OxH_2 and Ox standards, was spotted onto a silica gel TLC strip which was dried and developed with an ascending mobile phase consisting of butanol:pyridine:acetic acid:water in the volume ratio, 15:10:3:12. At 24 hours, a second aliquot of supernatant from the resin reaction was spotted on a silica gel strip and developed as above. The dried TLC strips were visualized by exposure to iodine vapor. OxH_2 had an Rf of 0.77 and Ox had an Rf of 0.52. The results indicated that after 5 min of exposure to Resin # 6, most of the OxH_2 had been converted to Ox, and after 24 hours of exposure, the conversion was complete.

EXAMPLE VII

Resin #7 has a DTNB group, the two carboxyl groups of which are amide-bonded to the α and ε amino groups of lysine

substituents on the resin. Each lysine molecule is amide-linked through its carboxyl group to an aminomethyl group which is attached to an aryl moiety of a styrene-divinylbenzene copolymer. This resin is similar to the DTNB-substituted resins in application.

Resin #7 is prepared as in the sequence of reactions as depicted by Figure 18. R_4 is styrene-divinylbenzene copolymer containing aminomethyl groups attached to aryl moieties of the resin. In the figure, -S... indicates a crosslink to a sulfur atom of a disulfide moiety of a like formula.

The production and analysis of one embodiment of Resin #7 is illustrated by the following experiment.

662 mg of N-α-N-ε-bisFmoc-lysine and 184 mg of HOBT were dissolved in 8 ml of 1-methyl-2-pyrrolidinone (NMP), and 187 μl of DIPCDI was added. The solution was mixed for 15 minutes at room temperature, 1 gm of aminomethylpolystyrene was added, and the suspension was mixed for 4 hours at room temperature. The resin was filtered, washed twice with alternating washes of NMP and methanol, twice with alternating washes of DCM and methanol and twice with DCM, and air dried. A sample of the resin gave a slightly positive ninhydrin test for primary amino groups. The resin was recoupled with bisFmoc-lysine as described above, except that the reaction was run overnight at room temperature. The resin was washed and dried as described above. A sample of the bisFmoc-lysine resin gave a negative ninhydrin test result. The Fmoc protecting group was removed from the lysine amino groups by adding the resin to 25 ml of 20 volume % piperidine in DCM and mixing the suspension for 1 hour at room temperature. The deprotected lysine resin was filtered, washed twice with alternating washes of DCM and methanol and twice with DCM and then air dried. 443 mg of DTNB and 343 mg of HOBT were dissolved in 10 ml of NMP, and 350 μl of DIPCDI was added. The deprotected lysine resin was added, and the suspension was mixed overnight at room temperature. The resultant DTNB resin

was filtered, washed twice with NMP, twice with methanol, twice with DCM and twice with alternating single washes of NMP and double washes of DCM. A ninhydrin test of a sample of the resin gave a positive result. The resin was recoupled overnight with DTNB as described above. The recoupled DTNB resin was filtered, washed twice with alternating washes of NMP and methanol and twice with DCM. The washed resin was air dried and then dried in vacuuo. The dried resin was added to excess acetic anhydride, and the suspension was mixed for 1 hour at room temperature to acetylate remaining amino groups on the resin. The "capped" DTNB resin was washed twice with alternating washes of DCM and methanol and twice with DCM and then air dried. A sample of the "capped" resin gave a negative result in the ninhydrin test for primary amines. 200 mg of DTNB resin was added to 2 ml of NMP containing 1 millimole of DTT. After mixing the suspension for 1 hour at room temperature, the resin and supernatant turned pink. The resultant 5-mercapto-2-nitrobenzoyl (MNB) resin was filtered, washed twice with alternating washes of NMP and methanol, once with alternating washes of DCM and methanol and twice with DCM. 50 mg of the air-dried red MNB resin was added to 1 ml of NaOH-saturated DMF (prepared by shaking 1 gm of NaOH with a solution of 1% water in DMF), and the suspension was mixed for 30 minutes at room temperature. 25 μl of a 1 M iodine in DMF was added to reoxidize the MNB to DTNB, and the suspension was mixed for 15 minutes at room temperature during which time the resin color changed from red to dull yellow. The DTNB resin was washed three times with alternating washes of DMF and methanol, once with an alternating wash of DCM and methanol and three times with DCM, and then air dried. A sample of the dried resin (Resin # 7) was resuspended in 400 μl of NMP, and a small amount of N-acetylcysteine was added. The resin color changed immediately from yellow to orange and the supernatant remained colorless, indicating that the DTNB substituent was

doubly linked to the resin and had been reduced to two resin-bound MNB groups. In the same procedure as described above in Example I, an aliquot of a solution of reduced oxytocin, OxH_2, in water was treated with a sample of Resin #7. After mixing the resin suspension for 1 hour, an aliquot of the supernatant was recovered and analyzed by TLC. From the observed Rf's, the resin effected an essentially complete conversion of OxH_2 to Ox.

EXAMPLE VIII

Resin #8 is identical with Resin #7 except that the DTNB group is replaced by a dithiodinicotinic acid (DTDNA) substituent.

Resin #8 is synthesized as shown in the sequence of reactions as depicted in Figure 19. Aminomethyl polystyrene is styrene-divinylbenzene copolymer containing aryl aminomethyl moieties, and DTDNA is 6,6'-dithiodinicotinic acid.

The production and analysis of one embodiment of Resin #8 is illustrated by the following experiment.

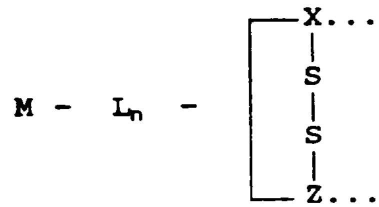
2 gm of aminomethyl resin (a matrix of styrene-divinylbenzene copolymer containing aryl aminomethyl groups) and 1.3 gm of N-α-N-ε-bisFmoc-lysine were added to 25 ml of DMF. 0.35 ml of DIPCDI was added, and the suspension was mixed overnight at room temperature. The bisFmoc-lysine resin was filtered and washed twice with alternate washes of DMF and methanol. A sample of the resin gave a positive ninhydrin test result, indicating incomplete coupling of bisFmoc-lysine to the aminomethyl resin. The resin was resuspended in 25 ml DMF, 1.3 gm of bisFmoc-lysine and 0.4 ml DIPCDI were added, and the suspension was mixed overnight at room temperature. The resin was filtered, washed twice with alternate washes of DMF and methanol and air dried. The bisFmoc-lysine resin was resuspended in a solution consisting of 20 ml of DMF and 5 ml of acetyl chloride, and the suspension was mixed for 1 hour at room temperature to acetylate unreacted amino groups on the

resin. The "capped" bisFmoc-lysine resin was filtered, washed three times with alternating washes of DMF and methanol and once with methanol and then air dried. The Fmoc protecting group was removed by adding the resin to 25 ml of 20 volume % of piperidine in DMF and mixing the suspension for 15 minutes at room temperature. The resultant lysine resin was filtered, washed twice with alternating washes of DMF and methanol and once with methanol and then dried in vacuuo. 1.6 gm of 6,6'-dithiodinicotinic acid (DTDNA) was dissolved in 20 ml of warm DMF, 0.7 ml of DIPCDI was added, the solution was mixed and added to the lysine resin and the suspension was mixed overnight at room temperature. The resultant DTDNA resin was filtered, washed twice with an alternating double wash of DMF and a single wash of methanol, then once more with methanol, and air dried. A sample of the pale yellow resin gave a negative ninhydrin test result, indicating complete coupling of the lysine amino groups to DTDNA. A sample of the resin was resuspended in 1 ml of DMF, and a small amount of DTT was added. Both the supernatant and the resin became bright yellow, indicating reduction of resin-bound DTDNA to 6-mercaptonicotinic acid moieties, of which one was released to the medium and one remained attached to the resin. 1 gm of the DTDNA resin was resuspended in 10 ml of a solution of 1.6 gm of N-acetylcysteine in DMF in order to reduce the DTDNA substituent to two MNA moieties. After mixing the suspension overnight at room temperature, the bright yellow suspension was filtered, washed twice with DMF, twice with alternating washes of methanol and DMF and twice with methanol and then air dried. The N-acetylcysteine reduction reaction was repeated on the MNA resin product, and the resultant resin was filtered, washed as above and dried in vacuuo over P_2O_5. The bright yellow MNA resin was oxidized with iodine in DMF containing sodium methoxide, washed and dried to give a pale yellow resin in which both nicotinic acid moieties of the dithio substituent

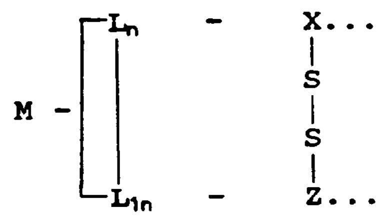
are attached to the resin. This was confirmed by suspending a sample of the DTDNA resin (Resin # 8) in DMF and adding a small amount of DTT. The resin color changed from pale yellow to bright yellow upon reduction of the DTDNA to two resin-bound MNA moieties, as indicated by lack of yellow color in the supernatant.

CLAIMS:

1. A composition comprising at least one disulfide moiety having the schematic Formula I



or the schematic Formula II



in which

M is a normally solid synthetic resin moiety;

L and L_1 are the same or different linker moieties;

n is zero or one; and

X and Z are the same or different disulfide substituted moieties each covalently joined to M or to a linker L or L_1;

wherein, following disulfide-thiol interchange, each of the resulting thiol moieties remains attached to the resin matrix moiety M.

2. The composition of claim 1 wherein

M is a hydroxyalkyl methacrylate-ethylene dimethacrylate copolymer; a methacrylic acid-hydroxypropyl methacrylate-ethylene dimethacrylate copolymer; a styrene-divinylbenzene crosslinked copolymer; an aminomethylated styrene divinylbenzene crosslinked copolymer; or a ω-aminopolyethylene glycol grafted styrene divinylbenzene crosslinked copolymer;

L and L_1 is lysine, ethylene diamine; tetraethylene glycol diamine ($H_2NCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$); triethylene glycol diamine ($H_2NCH_2CH_2OCH_2CH_2OCH_2NH_2$); or 1,1,1-tris(2-(2-aminopropyloxy)

propyloxymethyl)propane plus 1,1-bis(2-2-aminopropyloxy) propyloxymethyl)-1-(2-aminopropyloxymethyl)propane; and

X and Z moieties are 3-carboxypyridine-5-yl, 1-carboxy-2-nitrobenzene-5-yl, and 2-mercapto-1,3,4-thiadizole-5-yl, and are attached to L or M via the carboxyl or mercapto group.

3. Resin #1 of Figure 12 wherein R_1 is a hydroxyethyl methacrylate-ethylene dimethacrylate copolymer.

4. Resin #2 of Figure 13 wherein R_1 is a hydroxyethyl methacrylate-ethylene dimethacrylate copolymer.

5. Resin #3 of Figure 14 wherein R_1 is a hydroxyethyl methacrylate-ethylene dimethacrylate copolymer.

6. Resin #4 of Figure 15 wherein R_1 is a hydroxyethyl methacrylate-ethylene dimethacrylate copolymer.

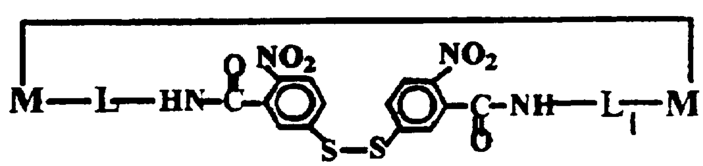
7. Resin #5 of Figure 16 wherein R_2 is a methacrylic acid-hydroxypropyl methacrylate-ethylene dimethacrylate copolymer.

8. Resin #6 of Figure 17 wherein R_3 is a ω-aminopolyethylene glycol (peg, n= @70) chains grafted onto styrene-divinyl benzene copolymer (1% divinylbenzene).

9. Resin #7 of Figure 18 wherein R_4 is aminomethylated 1% divinyl-benzene crosslinked polystyrene.

10. Resin #8 of Figure 19 wherein R_4 is aminomethylated 1% divinyl-benzene crosslinked polystyrene.

11. The compound having the schematic Formula III



in which

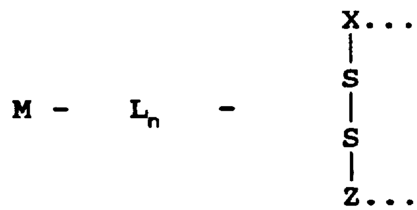
M is a normally solid synthetic resin moiety; and

L and L_1 are the same or different linker moieties.

AMENDED CLAIMS

[received by the International Bureau on 30 March 1995(30.03.95);
original claims 1 and 2 amended;
remaining claims unchanged (1 page)]

1. A composition comprising at least one disulfide moiety having the schematic Formula I



or the schematic Formula II

in which

M is any normally solid synthetic resin moiety;

L and L_1 are the same or different linker moieties;

n is zero or one; and

X and Z are the same or different disulfide substituted moieties each covalently joined to M or to a linker L or L_1;

wherein, following disulfide-thiol interchange, each of the resulting thiol moieties remains attached to the resin matrix moiety M.

2. The composition of claim 1 wherein

M is a hydroxylalkyl methacrylate-ethylene dimethacrylate copolymer; a methacrylic acid-hydroxypropyl methacrylate-ethylene dimethacrylate copolymer; a styrene-divinylbenzene crosslinked copolymer; an aminomethylated styrene divinylbenzene crosslinked copolymer; or a ω-aminopolyethylene glycol grafted styrene divinylbenzene crosslinked copolymer;

L and L_1 is lysine, ethylene diamine; tetraethylene glycol diamine ($H_2NCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$); triethylene glycol diamine; ($H_2NCH_2CH_2OCH_2CH_2OCH_2NH_2$); or 1,1,1-tris(2-(2-aminopropyloxy)

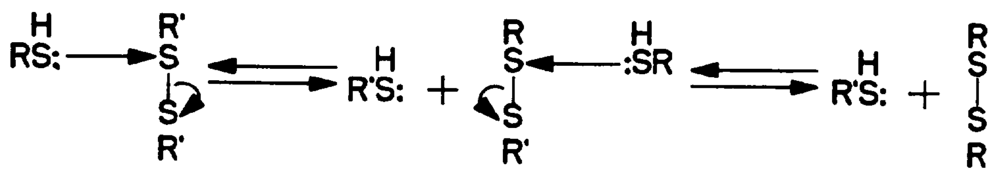


FIG. 1

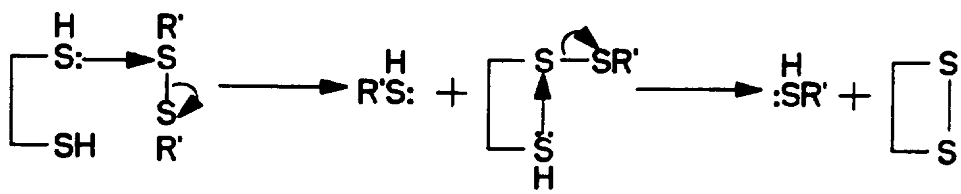


FIG. 2

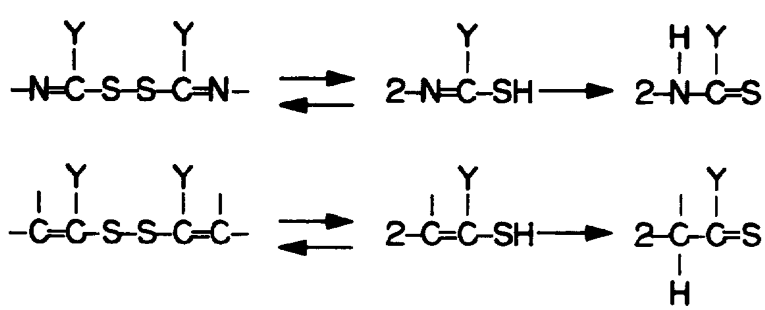


FIG. 3

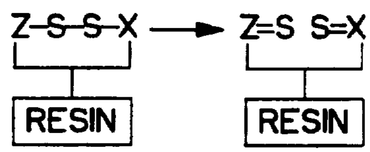

Z-S-S-X
Z=S S=X
RESIN
RESIN

FIG. 4

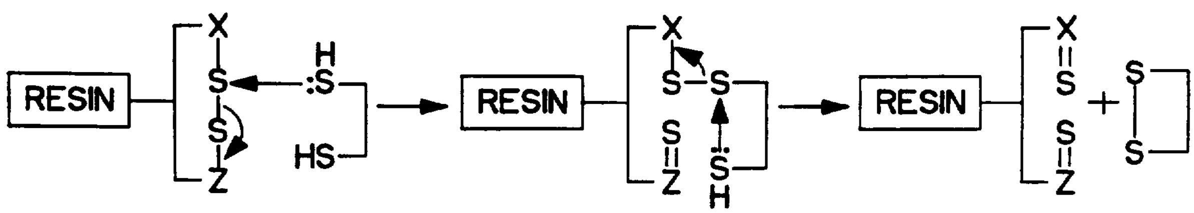
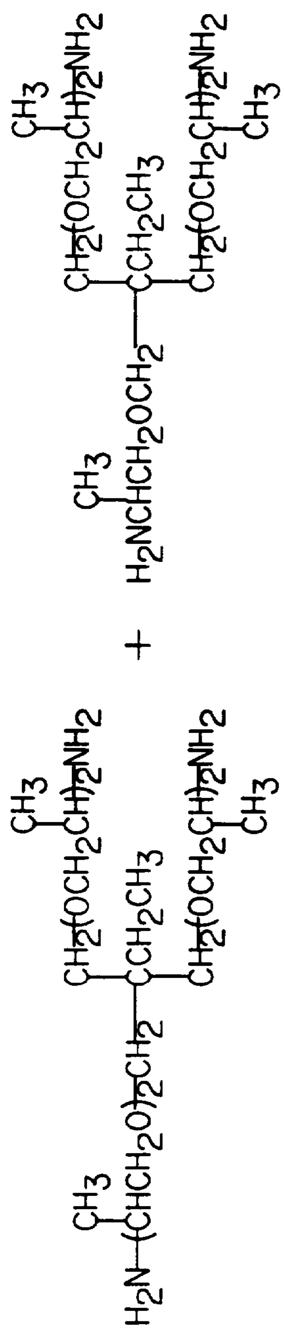

RESIN
X
S
S
Z
H
:S
HS
RESIN
X
S
S
S
Z
S
H
RESIN
X
S
S
Z
+
S
S

FIG. 5

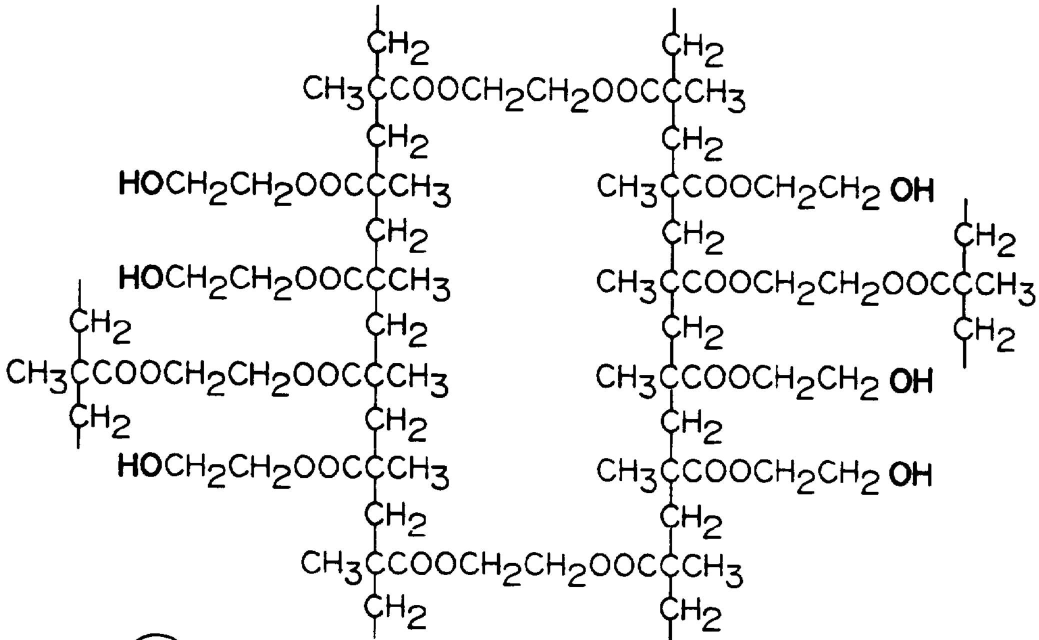


1,1,1-TRIS(2-(2-AMINOPROPYLOXY)PROPYLOXYMETHYL)PROPANE

1,1-BIS(2-(2-AMINOPROPYLOXY)PROPYLOXYMETHYL)-1-(2-AMINOPROPYLOXYMETHYL)PROPANE

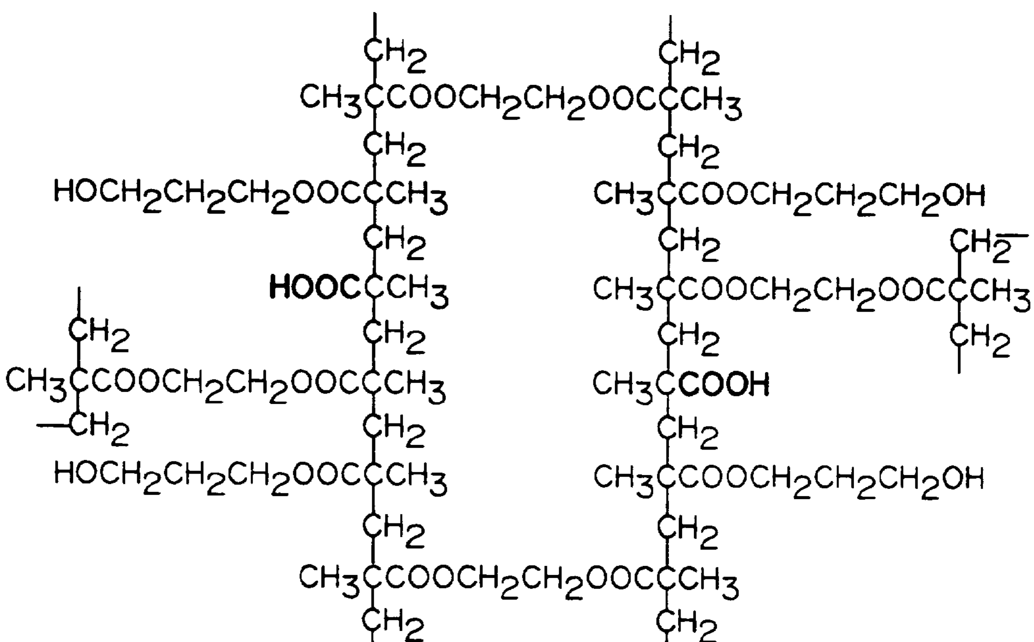
JEFFAMINE® T-403

FIG. 6



(R1)—OH = POLYHEMA(2.2 MILLIMOLES OH PER GRAM)
HYDROXYETHYL METHACRYLATE-
ETHYLENE DIMETHACRYLATE COPOLYMER

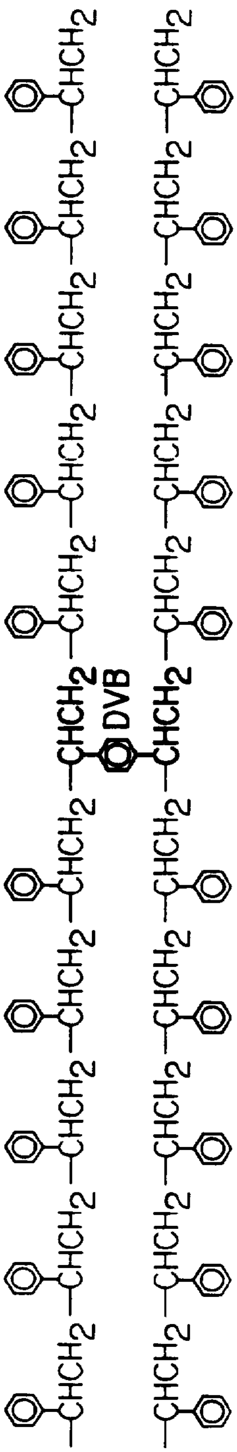
FIG. 7



(R2)—COOH = MACROPREP CM (@ 0.8 MILLIMOLES COOH PER GRAM)

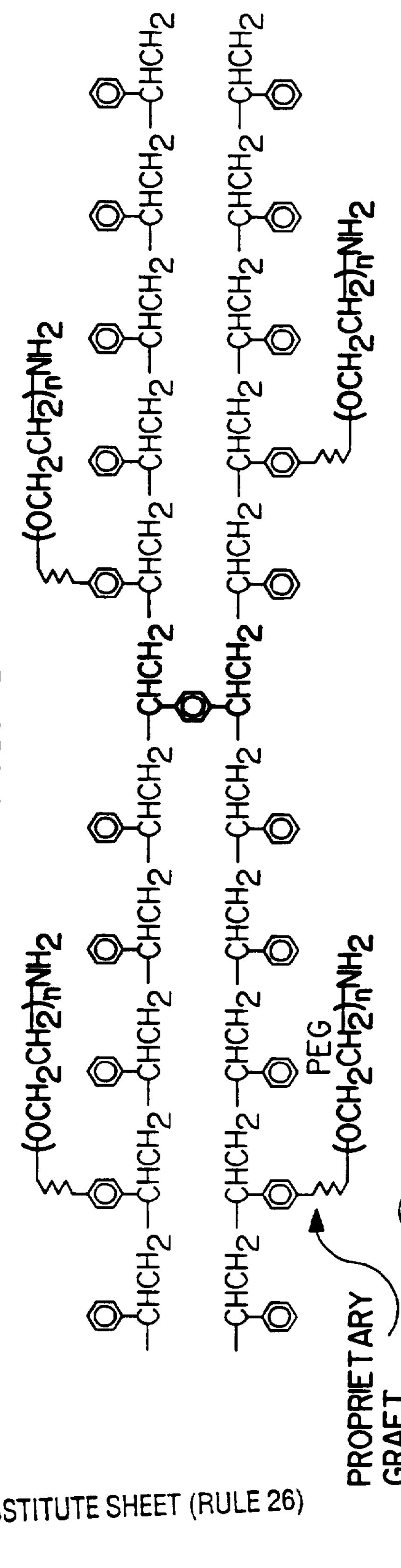
METHACRYLIC ACID/HYDROXYPROPYL METHACRYLATE
-ETHYLENE DIMETHACRYLATE COPOLYMER

FIG. 8



STYRENE-DIVINYLBENZENE (DVB) COPOLYMER (1% DIVINYLBENZENE)
COMMONLY CALLED 1% DVB- CROSSLINKED POLYSTYRENE

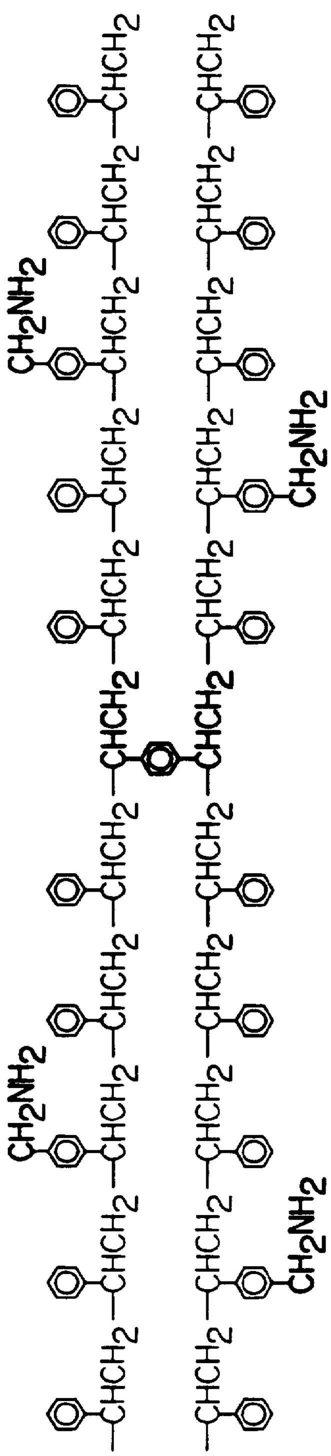
FIG. 9



(R3)—NH_2 = NOVASYN TG RESIN (@ 0.2 MILLIMOLES NH_2 PER GRAM)

ω-AMINOPOLYETHYLENE GLYCOL (PEG, n = @70) CHAINS GRAFTED ONTO STYRENE-DIVINYL BENZENE COPOLYMER (1% DIVINYLBENZENE)

FIG. 10



Ⓡ$_4$—NH_2 = AMINOMETHYLATED 1% DVB-CROSSLINKED POLYSTYRENE (0.56 MILLIMOLES NH_2 PER GRAM)

FIG. 11

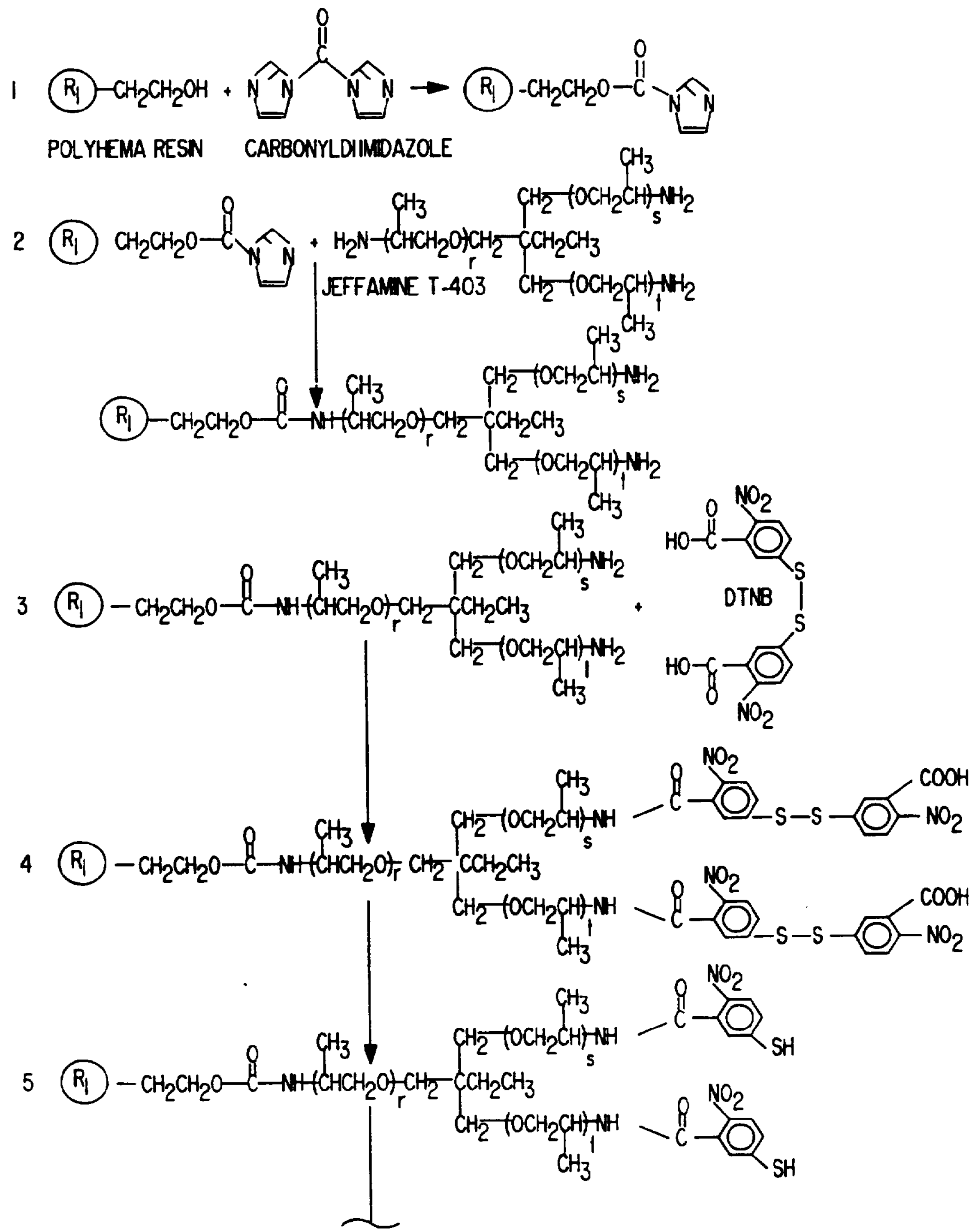

1
POLYHEMA RESIN
CARBONYLDIIMIDAZOLE
2
JEFFAMINE T-403
3
DTNB
4
5

FIG. 12A

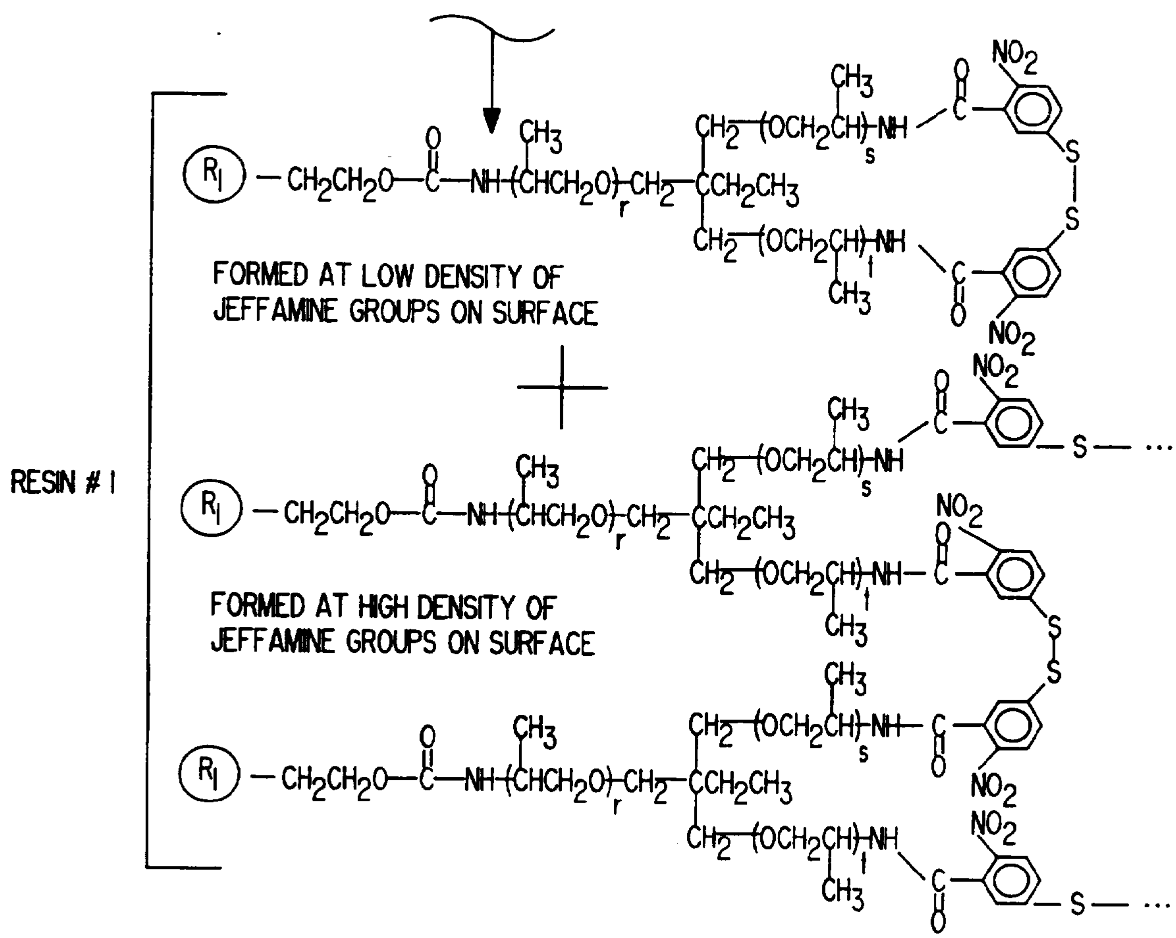
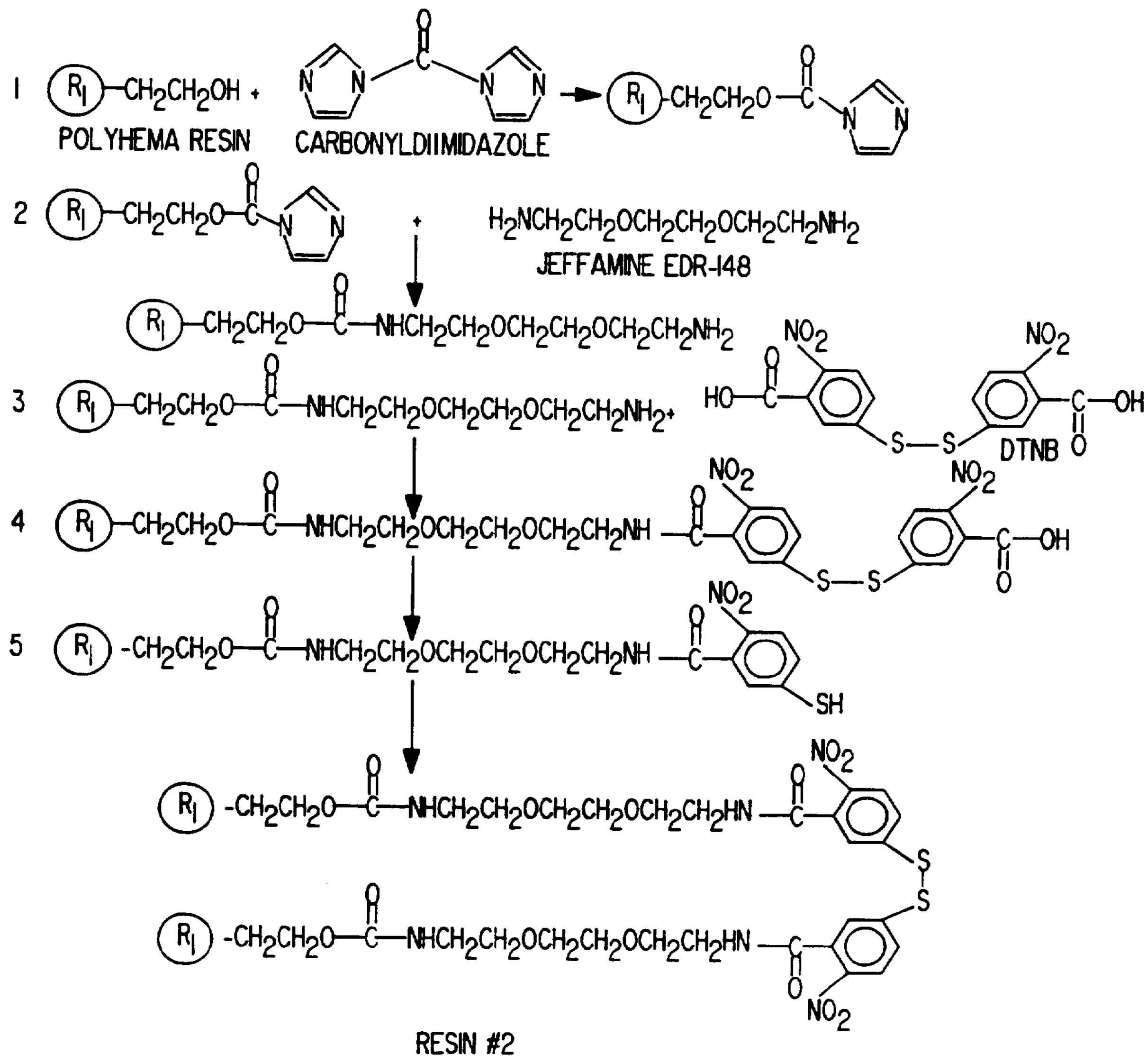
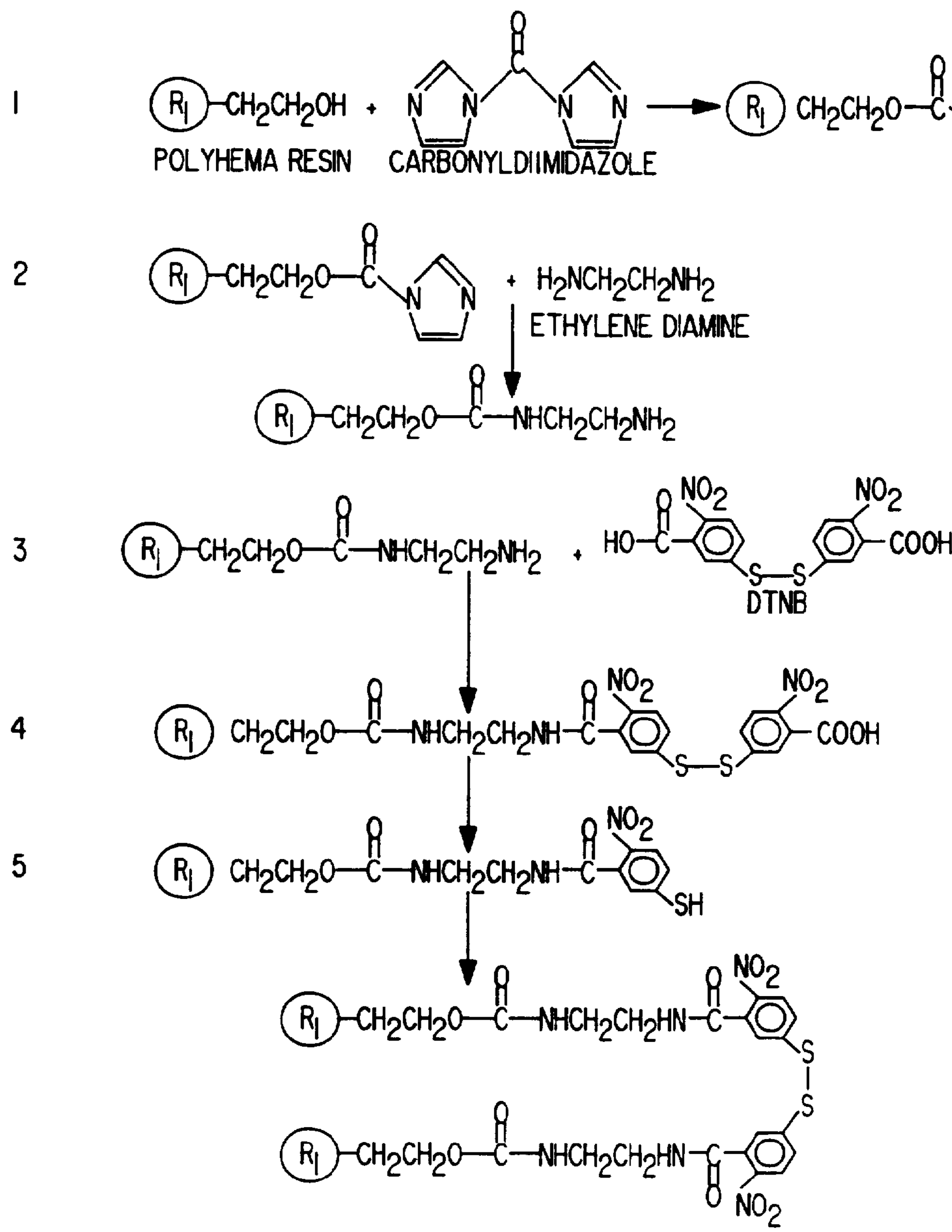

RESIN # I
FORMED AT LOW DENSITY OF JEFFAMINE GROUPS ON SURFACE
FORMED AT HIGH DENSITY OF JEFFAMINE GROUPS ON SURFACE

FIG. 12B



1
POLYHEMA RESIN
CARBONYLDIIMIDAZOLE
2
JEFFAMINE EDR-148
3
DTNB
4
5
RESIN #2

FIG. 13



RESIN #3

FIG. 14

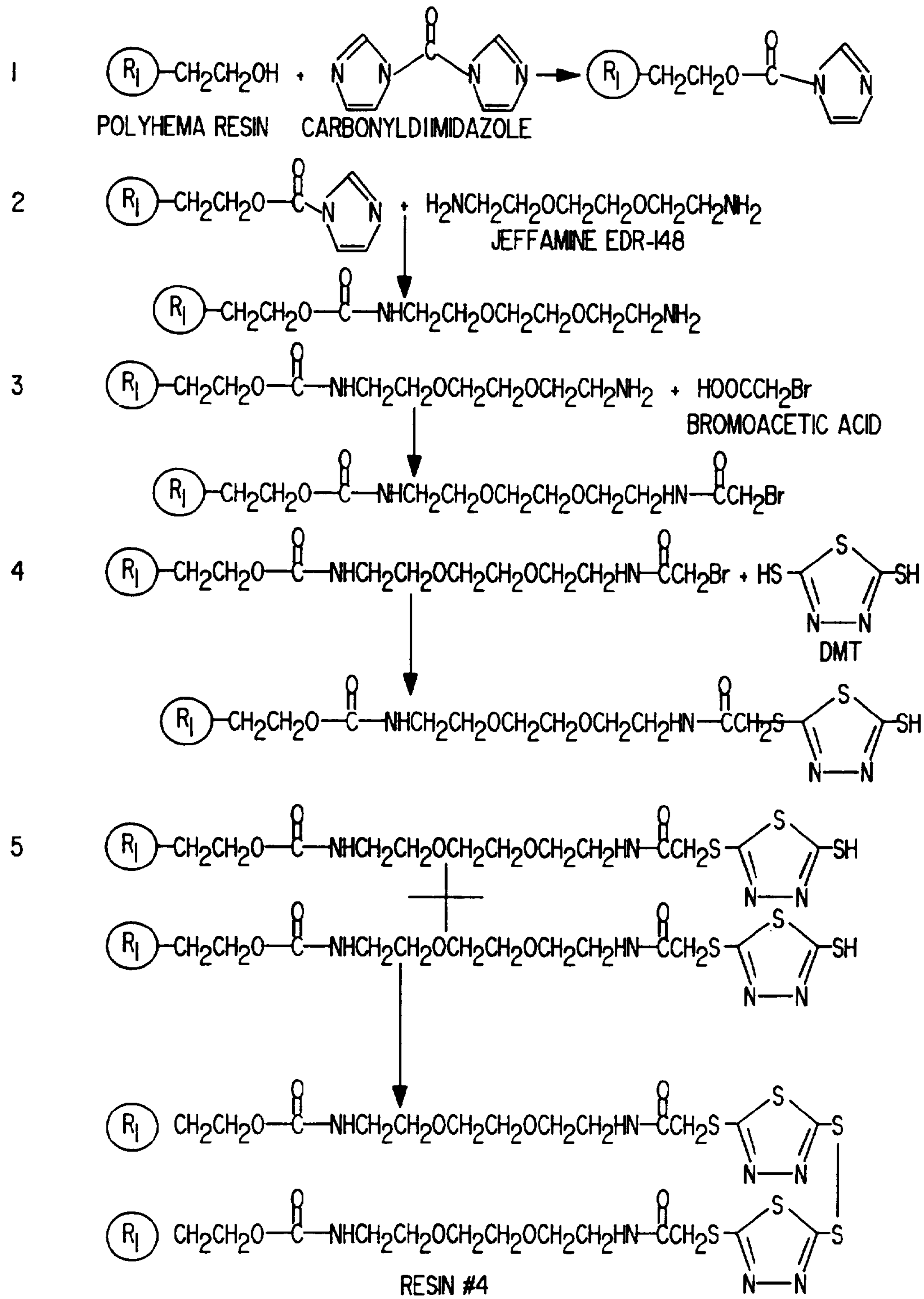
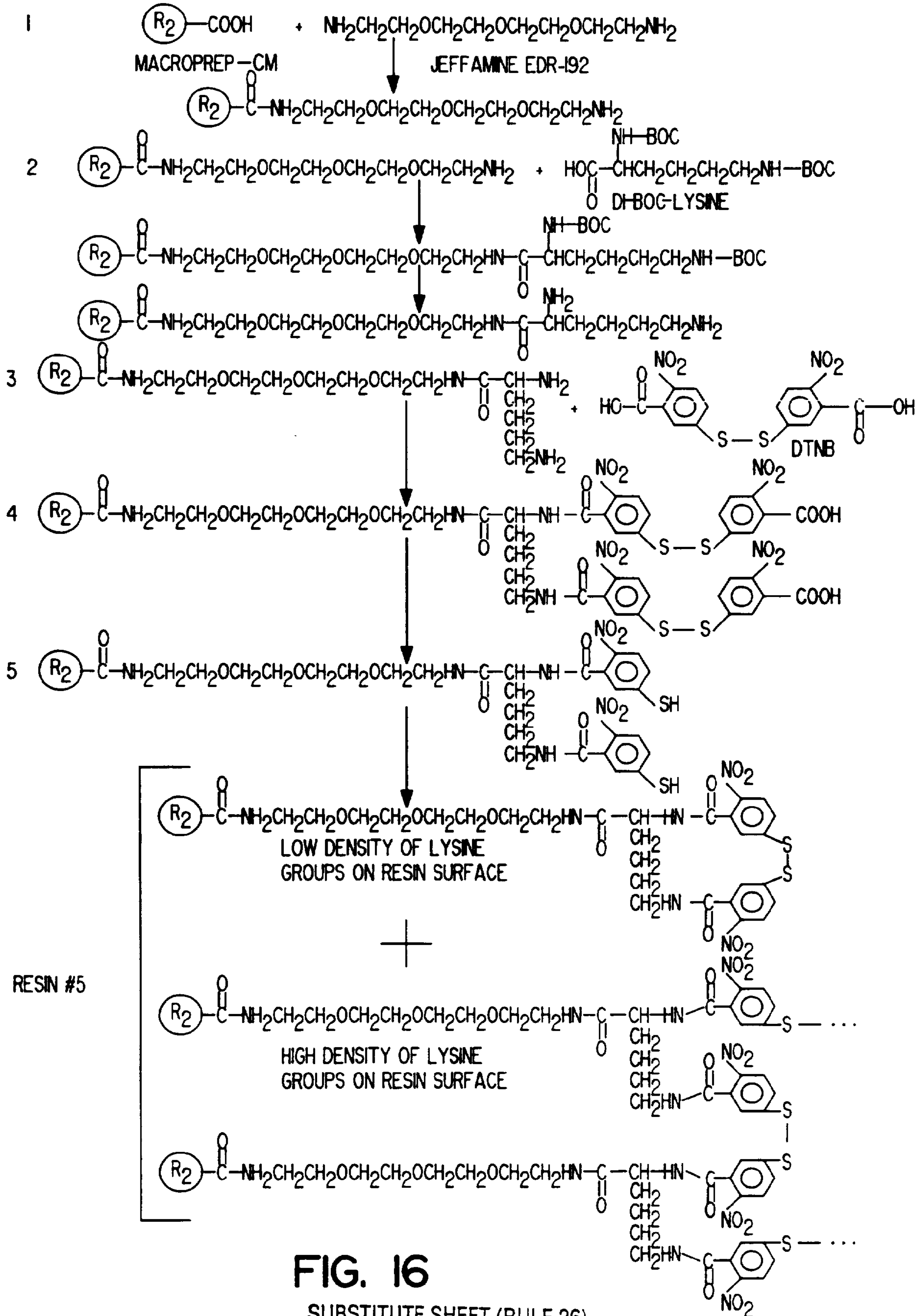

1
POLYHEMA RESIN
CARBONYLDIIMIDAZOLE
2
JEFFAMINE EDR-148
3
BROMOACETIC ACID
4
DMT
5
RESIN #4

FIG. 15



MACROPREP-CM
JEFFAMINE EDR-192
DI-BOC-LYSINE
DTNB
LOW DENSITY OF LYSINE GROUPS ON RESIN SURFACE
HIGH DENSITY OF LYSINE GROUPS ON RESIN SURFACE
RESIN #5

FIG. 16

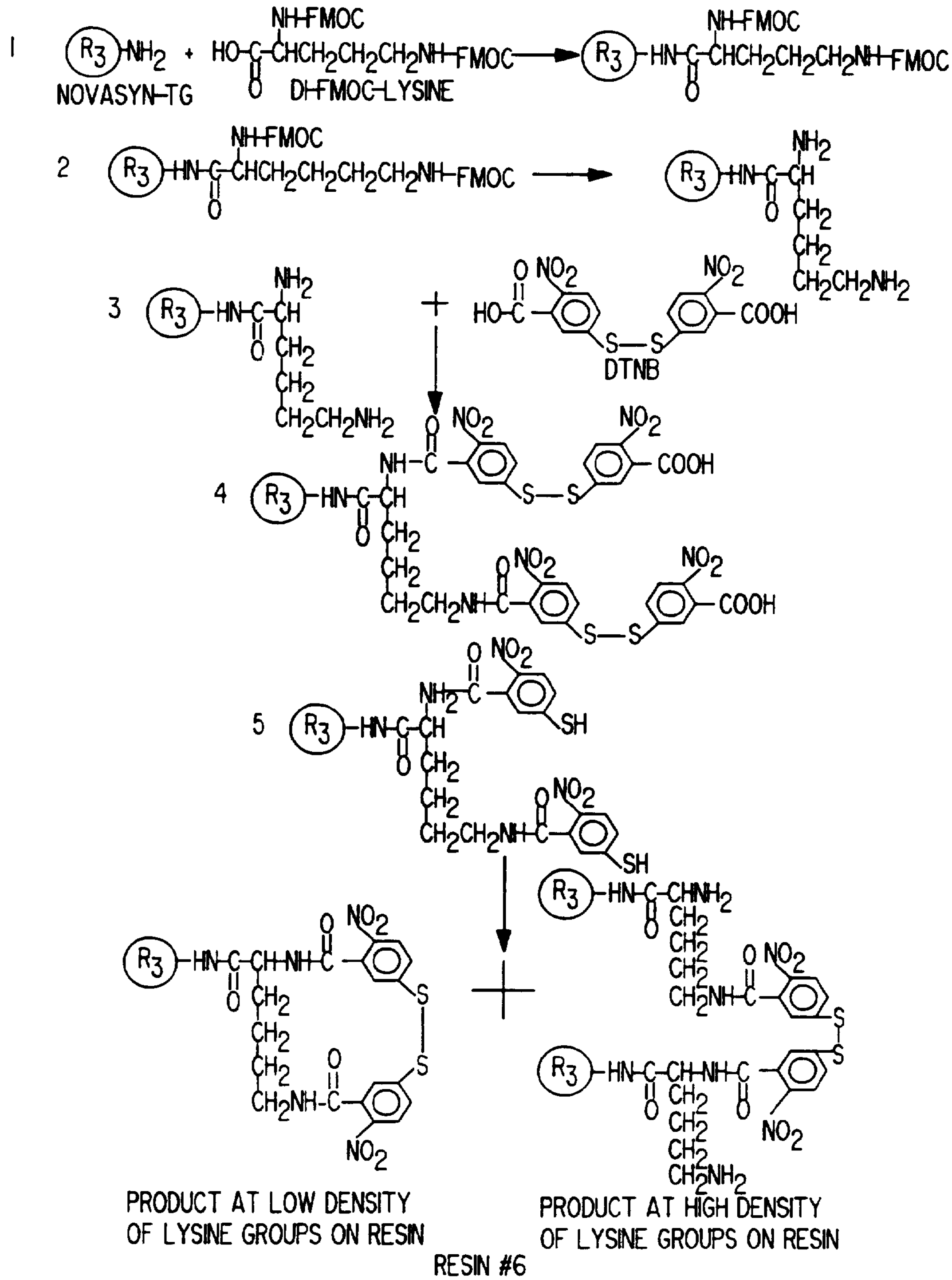
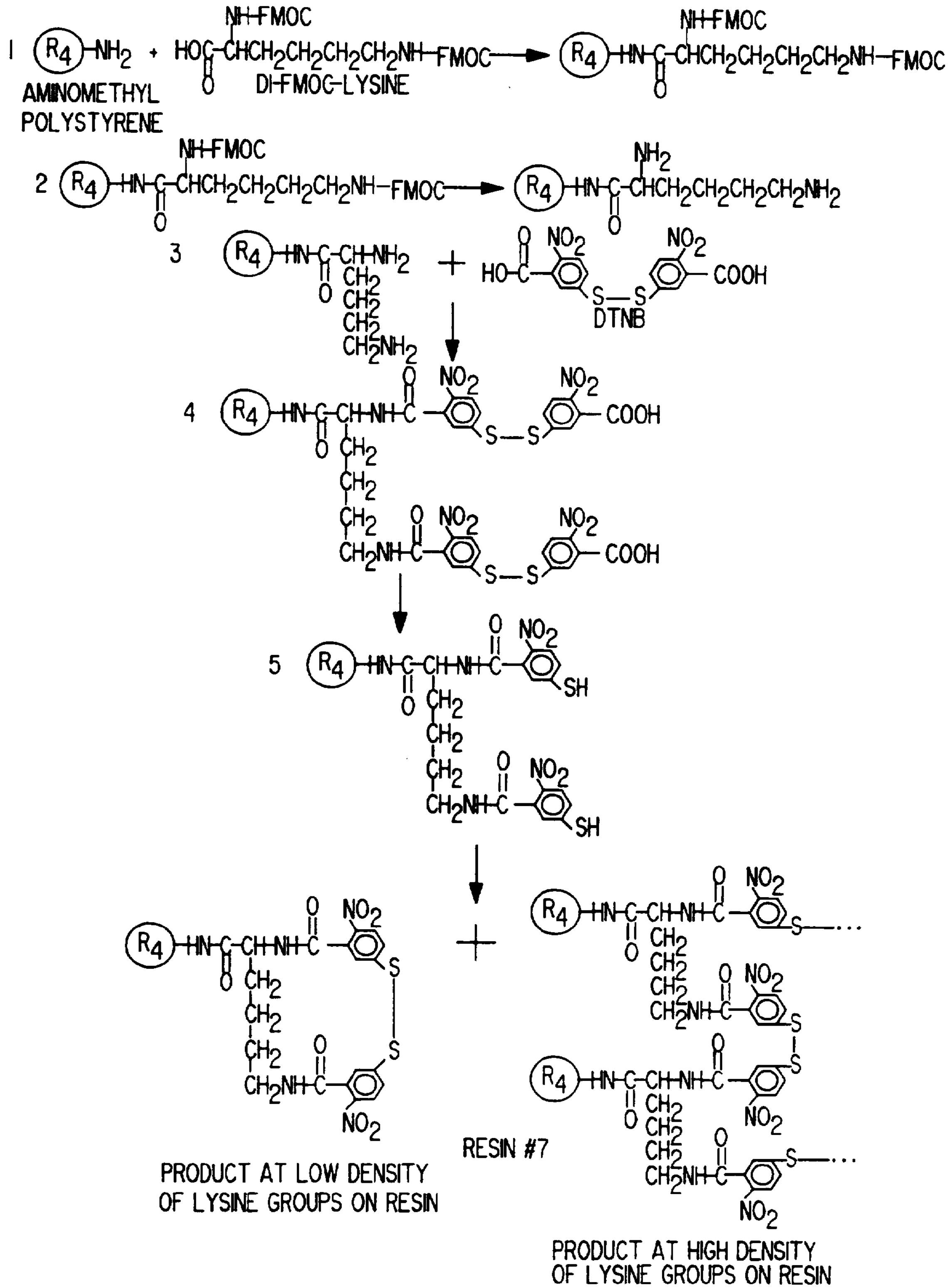

NOVASYN-TG
DI-FMOC-LYSINE
DTNB
PRODUCT AT LOW DENSITY OF LYSINE GROUPS ON RESIN
PRODUCT AT HIGH DENSITY OF LYSINE GROUPS ON RESIN
RESIN #6

FIG. 17



AMINOMETHYL POLYSTYRENE
DI-FMOC-LYSINE
DTNB
RESIN #7
PRODUCT AT LOW DENSITY OF LYSINE GROUPS ON RESIN
PRODUCT AT HIGH DENSITY OF LYSINE GROUPS ON RESIN

FIG. 18

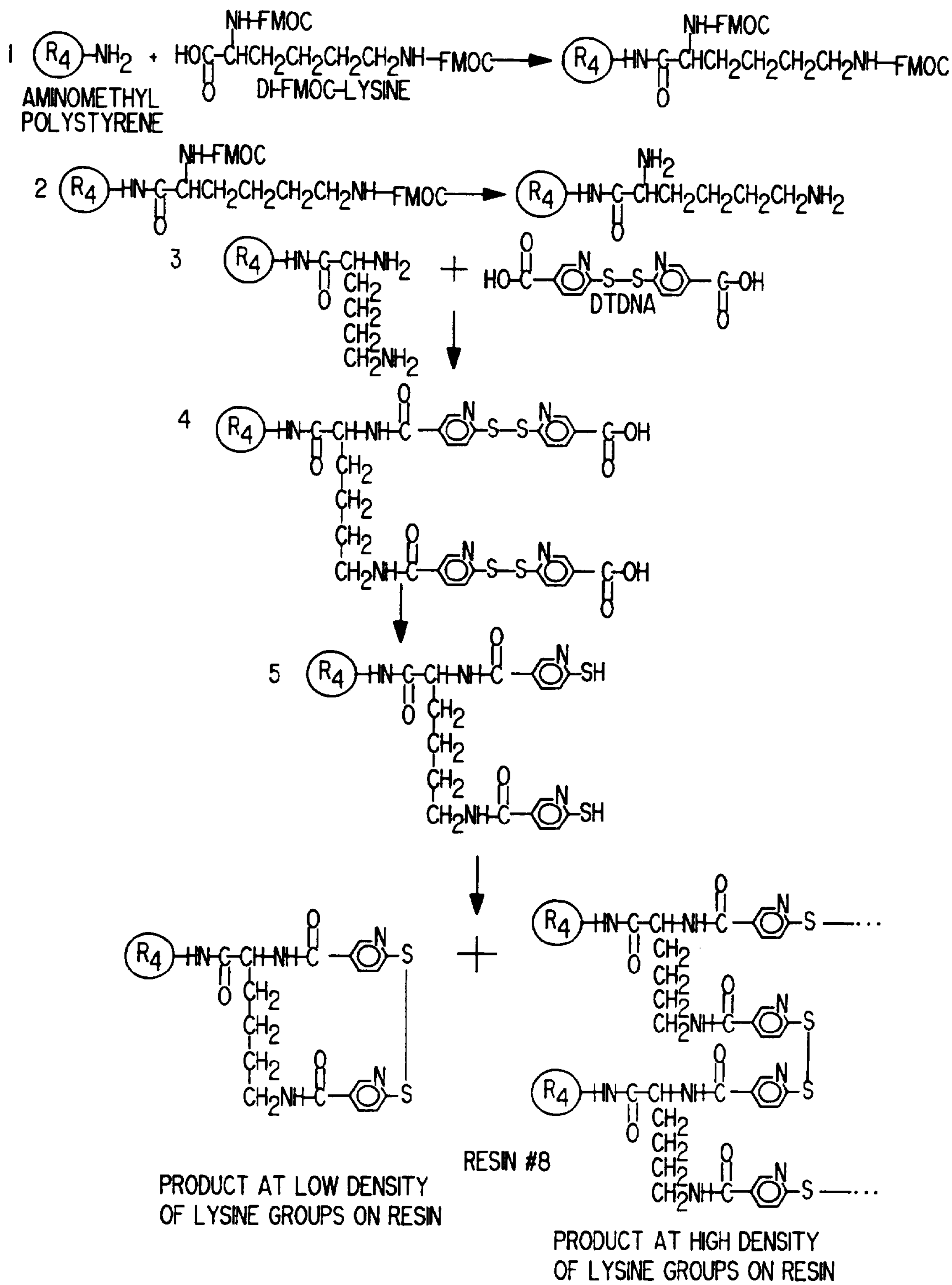

1
AMINOMETHYL POLYSTYRENE
DI-FMOC-LYSINE
NH-FMOC
2
3
DTDNA
4
5
RESIN #8
PRODUCT AT LOW DENSITY OF LYSINE GROUPS ON RESIN
PRODUCT AT HIGH DENSITY OF LYSINE GROUPS ON RESIN

FIG. 19

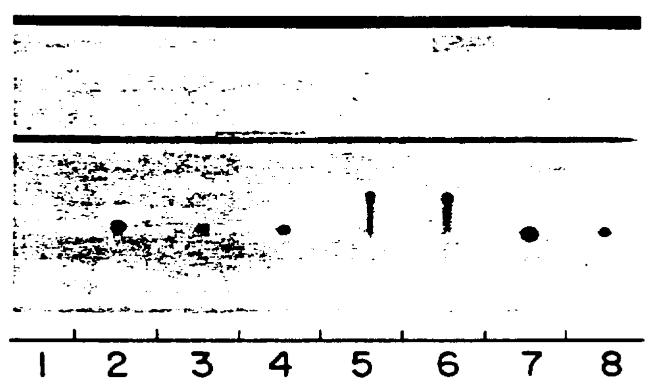


FIG. 20

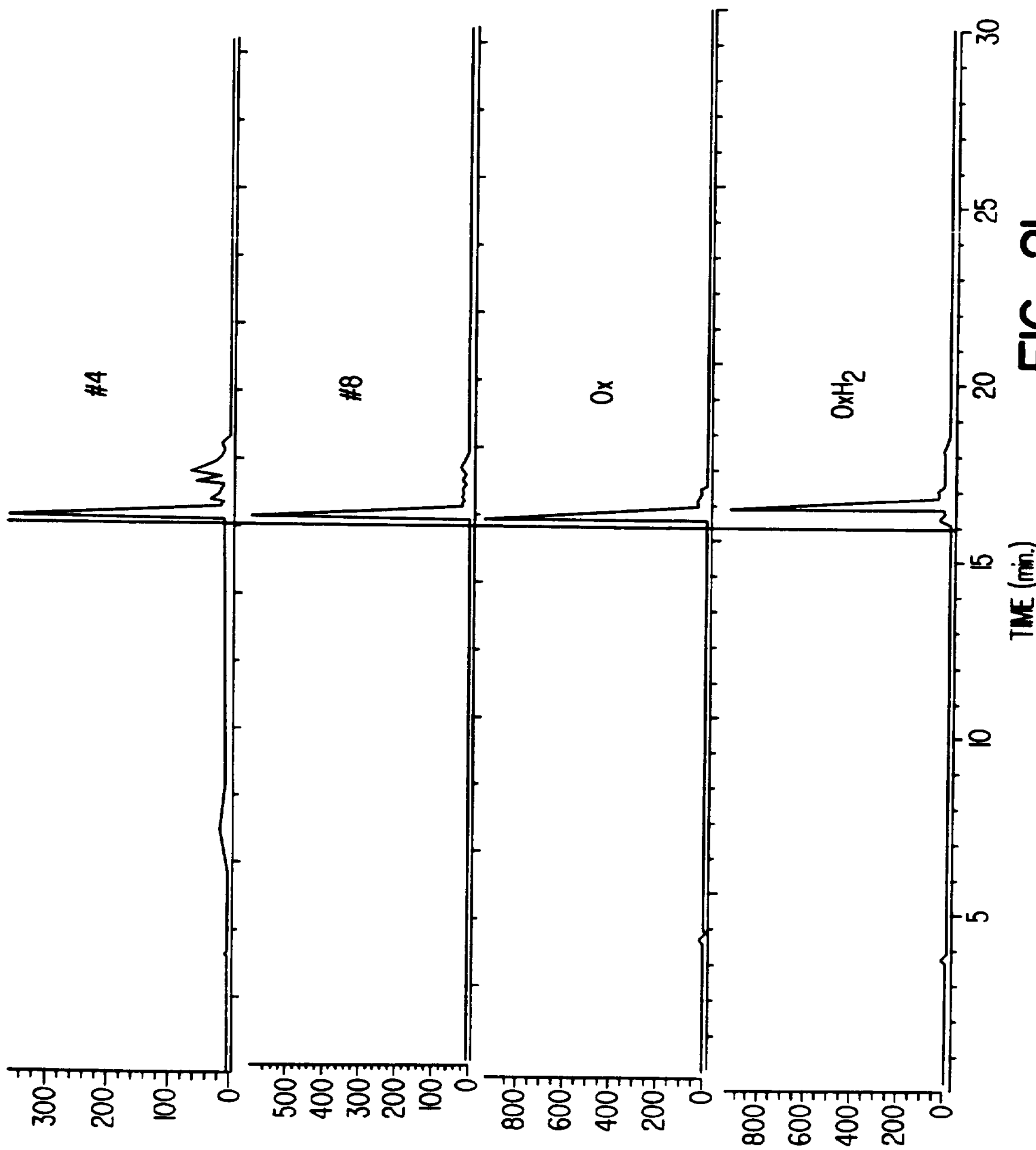

#4
#8
Ox
OxH2
TIME (min.)
5
10
15
20
25
30
300
200
100
0
500
400
300
200
100
0
800
600
400
200
0
800
600
400
200
0

FIG. 2I

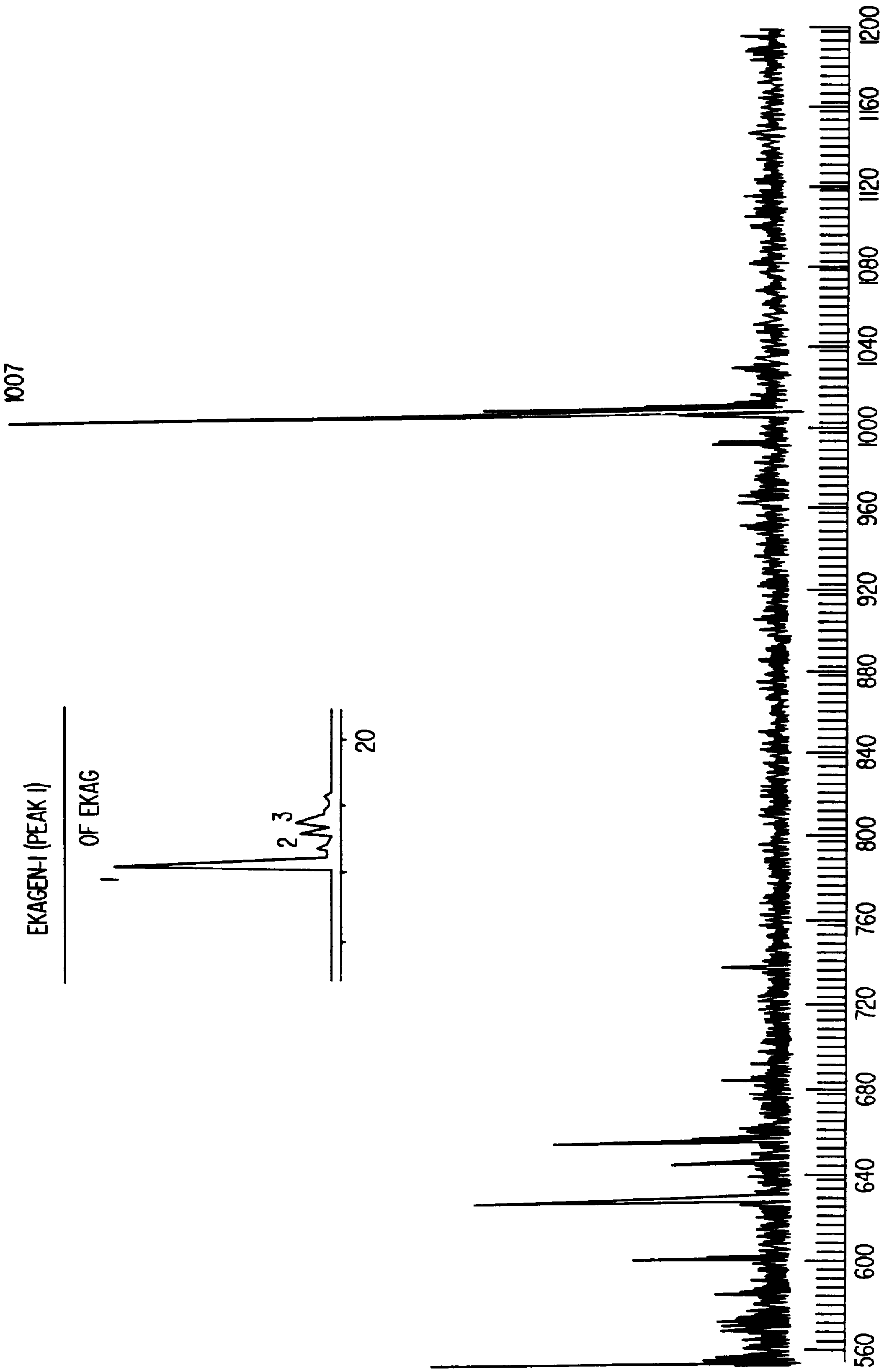

1007
EKAGEN-I (PEAK I)
OF EKAG
1
2
3
20
560
600
640
680
720
760
800
840
880
920
960
1000
1040
1080
1120
1160
1200

FIG. 22

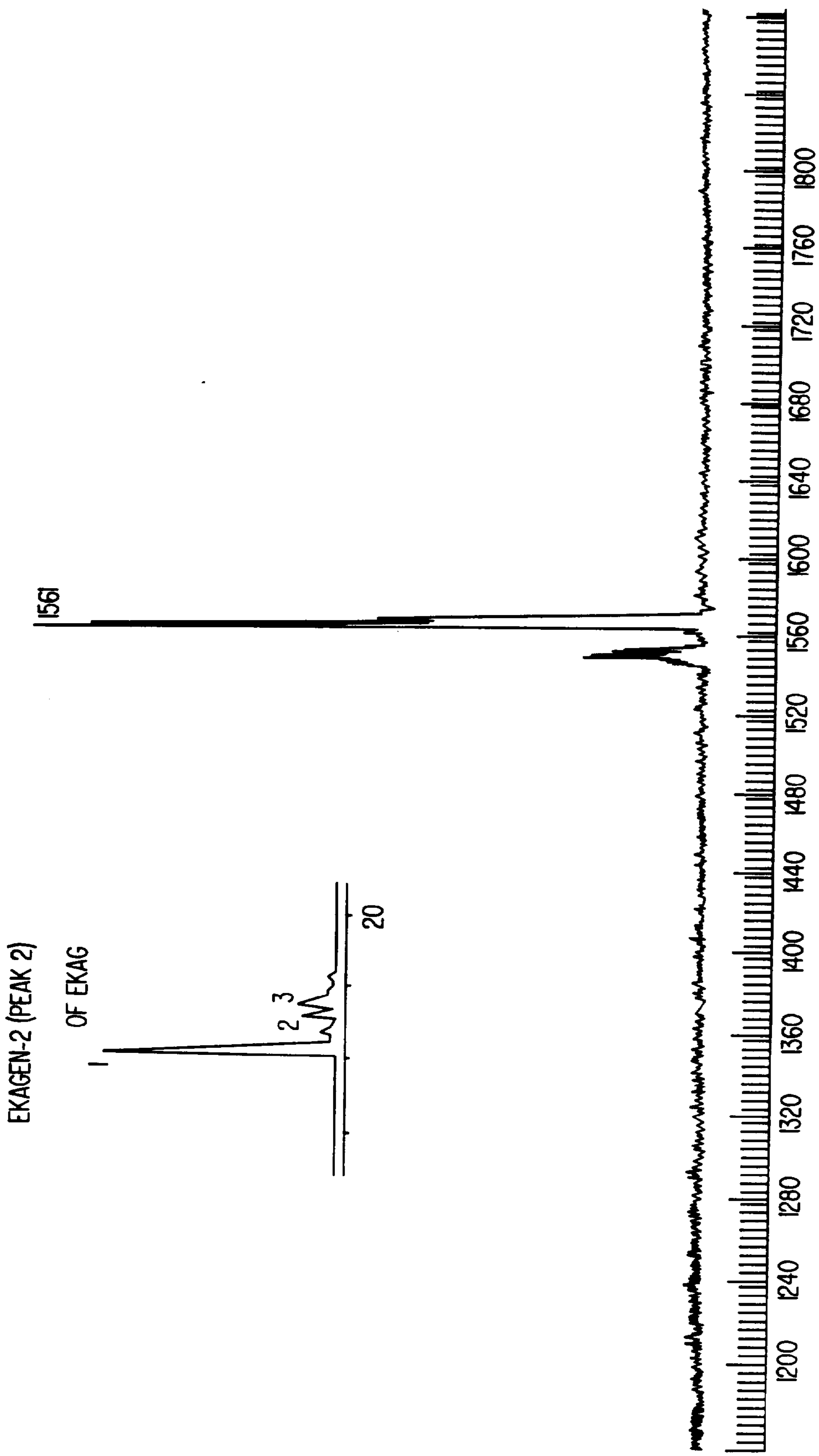

EKAGEN-2 (PEAK 2)
OF EKAG
1
2
3
20
1561
1200
1240
1280
1320
1360
1400
1440
1480
1520
1560
1600
1640
1680
1720
1760
1800

FIG. 23

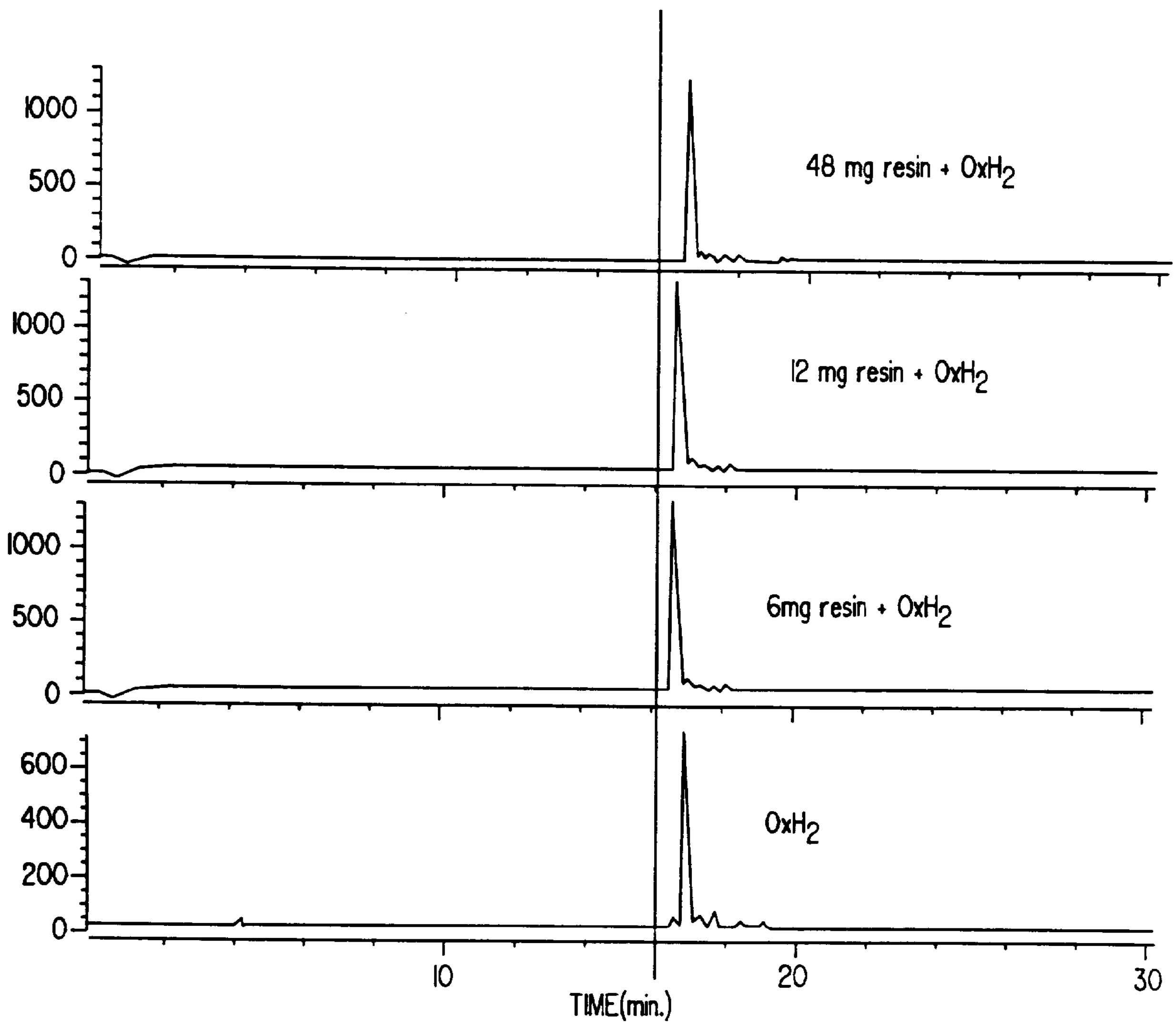

1000
500
0
48 mg resin + OxH2
1000
500
0
12 mg resin + OxH2
1000
500
0
6mg resin + OxH2
600
400
200
0
OxH2
10
20
30
TIME(min.)

FIG. 25

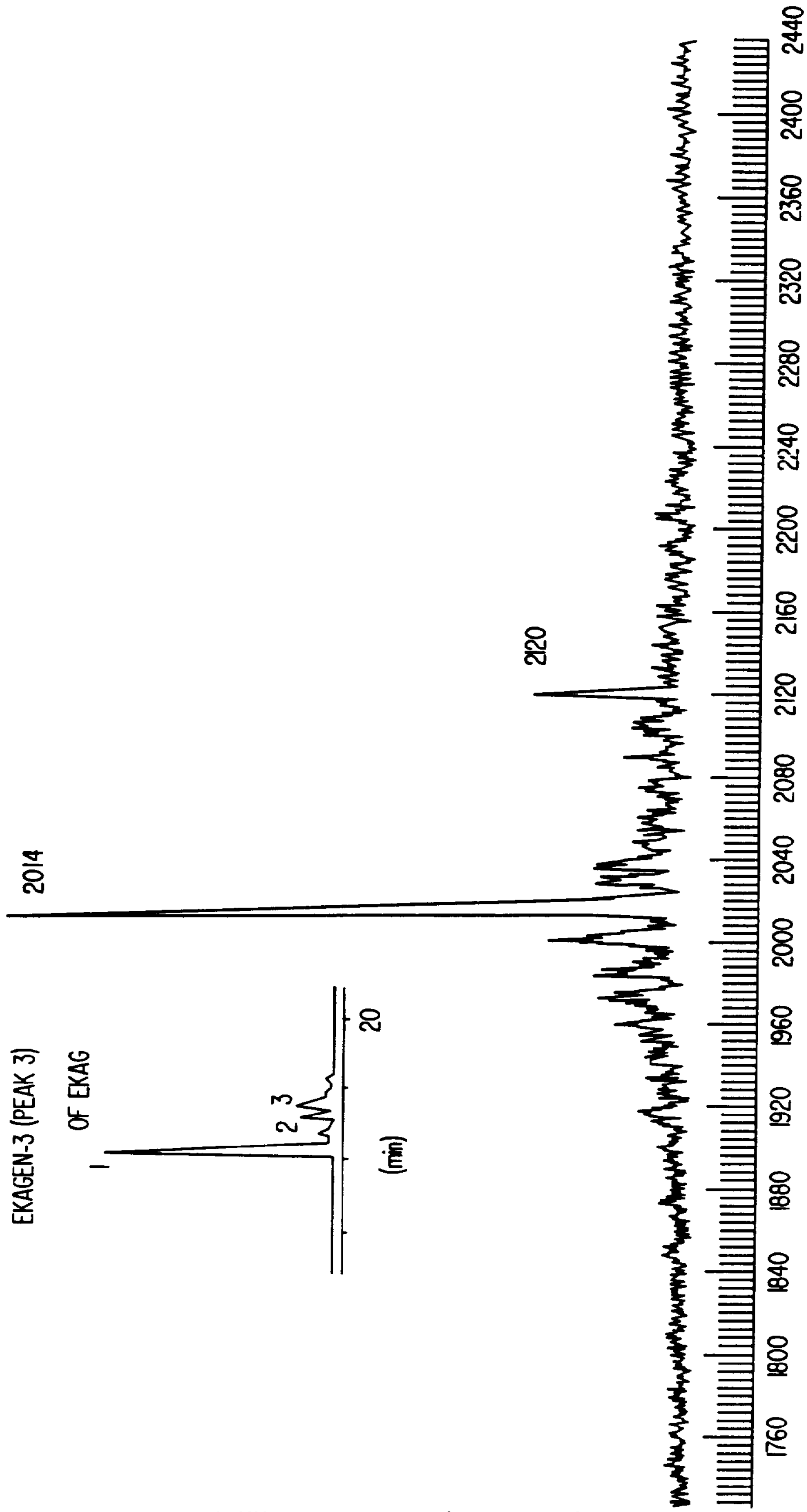

EKAGEN-3 (PEAK 3)
OF EKAG
1
2
3
20
(min)
2014
2120
1760
1800
1840
1880
1920
1960
2000
2040
2080
2120
2160
2200
2240
2280
2320
2360
2400
2440

FIG. 24

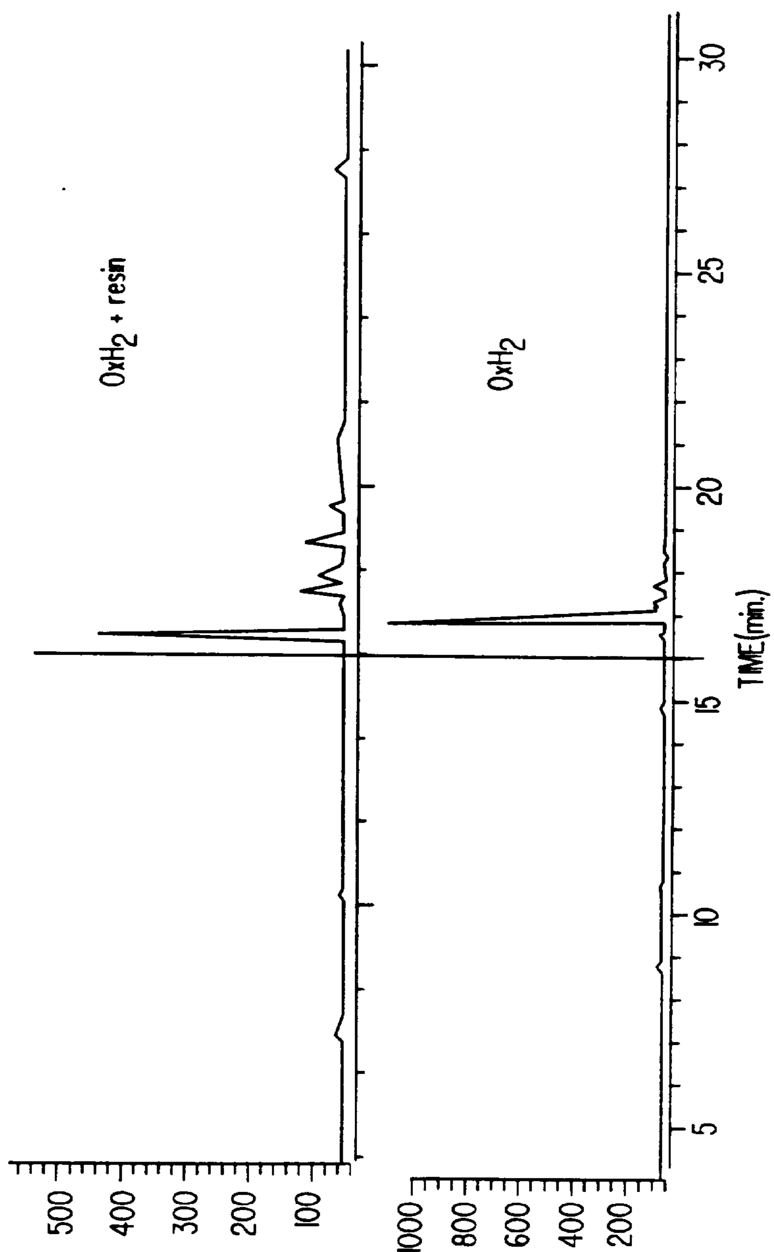

OxH$_2$ + resin
OxH$_2$
TIME(min.)
5
10
15
20
25
30
500
400
300
200
100
1000
800
600
400
200

FIG. 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10058

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C08F 8/34
US CL :525/328.8, 329.8, 330.4, 348, 349, 351
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 525/328.8, 329.8, 330.4, 348, 349, 351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 62-148949 (FUJI PHOTO FILM KK) 02 July 1987	1-11
A	US, A, 4,546,070 (KISHIMOTO et. al.) 08 October 1985	1-11
A	ANALYTICAL BIOCHEMISTRY, Vol. 134, (HARRIS et al), (1983), "Polyacrylamide Gels Which Contain a Novel Mixed Disulfide Compound Can Be Used to Direct Ensymes That Catalyze Thiol-Producing Reactions", pages 126-132.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 DECEMBER 1994	08 FEB 1995

Name and mailing address of the ISA/US	Authorized officer
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